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1) Appellant's Brief Under 37 C.F.R. § 41.37 (in triplicate)
2) Evidence Appendix – A (in triplicate)
3) Evidence Appendix – B (in triplicate)
4) Evidence Appendix – C (in triplicate)
5) Evidence Appendix – D1 (in triplicate)
6) Evidence Appendix – D2 (in triplicate)
7) Evidence Appendix – D3 (in triplicate)
8) Evidence Appendix – D4 (in triplicate)
9) Evidence Appendix – D5 (in triplicate)
10) Return Postcard

Melissa Hardy
Name

Melissa Hardy
Signature

IFW AF/1638

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Beyer, et al.	Art Unit: 1638
Serial Number: 09/914,913	Examiner: Russell Kallis
Filed: December 17, 2001	Confirmation No.: 5922
For: Method for Improving the Agronomics and Nutritional Value of Plants	Atty Docket: 60085 USPCT

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANT'S BRIEF UNDER 37 C.F.R. §41.37

This brief is filed under 37 C.F.R. §41.37 within two months of the filing of the Notice of Appeal, which was filed on September 19, 2006. Applicants/Appellants believe no fees are due in connection with this filing, however The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment, to Deposit Account No. 50-1744 in the name of Syngenta Biotechnology, Inc.

Real Party In Interest

The real party in interest in this matter is Syngenta Participations AG, per the assignment recorded in this case at Reel 012833, Frame 0959, on April 16, 2002.

Related Appeals and Interferences

None.

Status of Claims

Claims 1-15 are cancelled.

Claim 16 is rejected.

Claims 17-31 are cancelled.

Claims 32-43 are rejected.

Claims 44-59 are cancelled.

Claim 60 was newly presented in the Response to Final Office Action filed on August 15, 2006, but was not entered.

Status of Amendments

Appellants filed a Response to Final Office Action in this case on August 15, 2006, following a telephonic interview with the Examiner (summarized in Evidence Appendix, Exhibit A). This response was faxed to the Examiner, and the attached Auto-Reply Facsimile Transmission (Evidence Appendix, Exhibit B) was received by Appellants' Attorney. However, the Examiner has indicated that he did not receive the signed faxed copy (attached as Exhibit C of Evidence Appendix), and the Examiner mailed an Advisory Action and a Notice of Non-Compliant amendment (amendment unsigned) on September 29, 2006. Therefore, the amendments were not entered.

Summary of Claimed Subject Matter

Claim 16 is the only pending independent claim in the case. The claim relates to a method of producing plant cells that accumulate carotenoids (Figure 1 of the specification shows carotenoid biosynthesis), where the plant cells are normally carotenoid-free. The method involves transformation of plant material (page 15, line 25 – page 19, line 23) with DNA comprising an expression cassette capable of directing production in the plant cells of a phytoene synthase derived from a plant along with an expression cassette capable of directing production in the plant cells of a phytoene desaturase derived from a bacteria (page 21, line 1 – page 22, line 18). Transformed plant cells that accumulate carotenoids are then selected (page 23, line

10 – page 27, line 3). In the Response to Final Office Action discussed above (Exhibit C), Appellants had amended this claim to recite production of beta-carotene specifically, rather than carotenoids generally. Again, that amendment was not entered.

Grounds of Rejection to be Reviewed on Appeal

Claims 16 and 32-43 are rejected under 35 U.S.C.§102(b) as allegedly being anticipated by The Rockefeller Foundation, International Program on Rice Biotechnology; Workshop Report June 10-11, 1993, Potential for Carotenoid Biosynthesis in Rice Endosperm ("D1"), in light of Burkhardt P.K. et al. in Rice Genetics III; Proceedings of the Third International Rice Genomics Symposium; Khush G.S. Ed. 1996 (IRRI) International Rice Research Institute, pp. 818-820 ("D2") and in light of Ye X. et al. Science, 14 January 2000; Vol. 287, pp. 303-305 ("D3"). The Examiner takes the position that D1 describes methods for producing rice cells that accumulate carotenoids by transformation with various genes, particularly the entire beta-carotene pathway of *E. herbicola*. With respect to D2, the Examiner states that the reference describes rice transformed with the phytoene synthase and phytoene desaturase from daffodil, and describes phytoene accumulation. The Examiner states that D3 teaches transgenic rice with a plant phytoene synthase and a bacterial phytoene desaturase. The Examiner's conclusion is that D1 therefore inherently teaches a method of producing plant cells that accumulate carotenoid by transformation with a gene from daffodil encoding a phytoene synthase and a gene from daffodil encoding a phytoene desaturase of by transformation with a gene from daffodil encoding a phytoene synthase and a bacterial *crtI* gene from *E. uredovora* encoding a phytoene desaturase and plant cells and plants transformed thereby (Final Office Action at page 5).

Claims 16 and 32-43 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over D2, in view of Bramley P.M., Pure and Appl. Chem., 1997; Vol. 69, No. 10; pp. 2159-2162 ("D4"). The Examiner has taken the position that the teaching of D2 in view of D4 would have lead one of ordinary skill in the art to have a reasonable expectation of success in transforming a rice plant with a plant phytoene

synthase and a bacterial phytoene desaturase, leading to production of provitamin A or beta carotene in the endosperm of rice (Final Office Action, pages 6-7).

Appellants note that all of the references cited by the Examiner, along with D5 (presented by Appellants) are included in the Evidence Appendix.

Argument

Claim Rejections Under 35 U.S.C. §102

Appellants have taken the position that D1 is not anticipatory, in that the reference does not disclose every element of the claimed invention arranged as in the claim under review.

The Examiner has disputed Appellants' assertion that D1 represents early stage discussions and experiments by experts in the field in assessing the potential for carotenoid production in rice. The Examiner stated that D1 recites "Amidst optimism that the goal of accumulating nutritionally significant levels of carotenoid synthesis could be achieved the meeting was adjourned".

However, Appellants believe that D1 sets out two specific "strategies" for providing carotenoid accumulation in the rice endosperm: (1) a "non-green option" (comprising a bacterial phytoene synthase and a bacterial phytoene desaturase); and (2) a "mixed option" (comprising a plant phytoene synthase and a plant phytoene desaturase). Although D1 does discuss both plant and bacterial genes, it does not specifically disclose a method comprising a combination of a plant phytoene synthase and a bacterial phytoene desaturase. The Examiner has combined separate and distinct elements disclosed in D1 in order to arrive at the present invention, but it is well settled in the law that for a reference to anticipate under 35 U.S.C.§102, every element of the claimed invention must be identically shown in a single reference, and the elements must be arranged as in the claim under review.

Appellants realize that it is also well settled that the legal premise just set out is not "*ipsissmis verbis*" (is not "expressly... in words"), so that D1 could be

considered anticipatory if a person of ordinary skill in the art would understand the reference as disclosing all elements, in proper combination or arrangement, based on their own knowledge and the state of the art. However, in this regard Appellants have submitted document D5, submitted to the Examiner and herewith (Evidence Appendix, Reference D5), which provides a history of "Golden Rice" (to which the present invention relates). The author of D5 is Professor Ingo Potrykus, who is named as a co-inventor in respect of the present application and who was present at the workshop summarised in D1.

On page 1158 of D5 it is stated "the verdict of this initial session [the Rockefeller workshop] was that such a project had a low probability of success, but that it was worth trying because of its high potential benefit" (emphasis added). Furthermore, it is stated "There are hundreds of scientific reasons why the introduction and coordinated function of these enzymes would not be expected to work. Those with the necessary scientific knowledge were right in not believing in the experiment" (emphasis supplied). Indeed, even reference D1 concludes, *inter alia*, that "it is likely that beta-carotene accumulation in the rice endosperm will require the introduction of the complete set of genes coding for phytoene synthase onwards" (Appendix D of D1). In addition, D5 highlights the scepticism that existed at the time of D1 concerning the feasibility of successfully engineering an entire biosynthetic pathway into rice. The expectation of success in achieving the desired goal was thus extremely low.

However, contrary to the suggestion of D1, the methods of the present invention do not require a "complete set" of carotenoid biosynthetic genes in order to produce β-carotene in the rice endosperm. Indeed, it came as a surprise that β-carotene, rather than lycopene, accumulation was achieved via the use of only a plant phytoene synthase and a bacterial phytoene desaturase (as evidenced in reference D3, which is discussed further below) in the absence of engineering into the rice plant a lycopene cyclase. Thus, D1 can be seen to teach away from the present invention. Although D1 does recite a plant phytoene synthase and a bacterial phytoene desaturase, it contains no suggestion or motivation to combine these distinct features in order to provide a method to accumulate β-carotene in the rice endosperm.

Thus, although D1 suggests that there may have been "optimism" that significant levels of carotenoid synthesis could be achieved – there actually existed significant doubt among those of skill in the art (as discussed in D5) that a result would actually be achievable. At best, D1 can be seen as an invitation to carry out further research – the reality being that the methods of the present invention for providing β-carotene accumulation in the rice endosperm (the language adopted in new claim 60) actually took many years to achieve. This, combined with the fact that D1 does not disclose the claimed combination of a plant phytoene synthase with a bacterial phytoene desaturase, leads to the conclusion that D1 is not anticipatory under 35 U.S.C. §102(b).

The Examiner has also relied on reference D3, Ye et al., in arriving at the conclusion that "The Rockefeller reference teaches inherently, a method of producing plant cells that accumulate carotenoid by transformatino with a gene from daffodil... encoding a phyotene synthase and a gene from daffodil... encoding a phyotene desaturase or by transformation with a gene from daffodil encoding a phytoene synthase and a bacterial *crtI* gene... encoding a phytoene desaturase and plant cells and plants transformed thereby...." However, the foreign priority document to which the present application claims priority was filed on March 5, 1999, and D3 was published in the 14 January 2000 issue of Science. Furthermore, D3 represents the work of Beyer and Potrykus as disclosed in the present application, and thus could not have appeared before the making of the claimed invention. In any event, the publication of D3 occurred well after the priority date, and less than one year from the §371 international filing date (March 3, 2000), and therefore D3 is not available as a reference under either 35 U.S.C.§102(a) or (b). In any event, Appellants submit that it is improper for the Examiner to rely on D3 (published in 2000) in combination with D1 (1993) and D2 (1996) to support the alleged inherent disclosure of an invention made at least on, or before, March 5, 1999, and well before the publication of D3.

Therefore, for the foregoing reasons it is respectfully submitted that the subject matter of the presently submitted claims is in fact novel over D1, and Appellants respectfully request that this rejection be withdrawn.

Claim Rejections Under U.S.C §103

Claims 16 and 32-43 are rejected under 35 U.S.C.§103 as allegedly being obvious over D2 and D4. The Examiner suggests that, in view of D2 and D4, "one would have had reasonable expectation of success in transforming a rice plant with a plant phytoene synthase and a bacterial phytoene desaturase; and in producing provitamin A or beta carotene in the endosperm of rice". Respectfully, Appellants strongly disagree with this assertion, for the following reasons.

As discussed above with regard to D1, D2 suggests (page 819) that four enzymes are necessary for β-carotene biosynthesis in the rice endosperm (phytoene synthase, phytoene desaturase, ζ-carotene desaturase and lycopene cyclase). Thus D2, like D1, teaches away from the claimed method for reasons already stated above.

D2 also discloses a transgenic rice plant purported to comprise a plant (daffodil) phytoene synthase and a plant (daffodil) phytoene desaturase. It is reported that the transgenic rice plant accumulated a single carotenoid (phytoene) but not ζ-carotene, the product of phytoene desaturase (page 820).

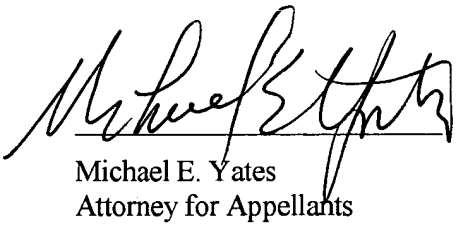
The work reported in D2 was important since it indicated that was possible to produce phytoene in the rice endosperm (indeed this was considered to be the "first breakthrough" in the development of Golden Rice - D5 page1158). However, D2 does not solve the problem of the production of carotenoids other than phytoene in the rice endosperm – since the phytoene desaturase that was introduced did not result in the accumulation of ζ-carotene as expected. Furthermore, D2 does not teach, or even suggest, a method by which β-carotene can be produced in the rice endosperm. There is certainly no teaching or suggestion in D2 that β-carotene can be produced in the rice endosperm by replacing the plant phytoene desaturase with a bacterial phytoene desaturase. Indeed, the skilled person would understand from D1 that the use of bacterial carotenoid gene(s) to produce carotenoids in plants is not ideal – since, while they appear to be functional, they can result in non-viable plants (D1 - page 3).

Bramley *et al.* (D4) disclose, *inter alia*, the use of a bacterial phytoene desaturase gene (*crt* I) to increase carotenoid levels in tissues (tomato fruit) which already have the capacity to synthesis high levels of carotenoids. Thus the skilled person would appreciate that, in the methods taught by Bramley, all of the biosynthetic machinery required to produce carotenoids are already present in the tissues transformed with the *crt*I gene. D4 is thus not concerned with the problem addressed by present invention – that is the accumulation of carotenoids in tissues which are normally carotenoid free.

There is no suggestion in either D2 or D4 that β-carotene accumulation could be achieved in the rice endosperm by the use of a plant phytoene synthase and a bacterial phytoene desaturase (e.g *crt* I) alone. Although it is possible to combine the teachings of D2 and D4 and arrive at the present invention there is absolutely no motivation to do so in the absence of the present invention. In doing so, it is submitted that the Examiner has made an *ex-post facto* analysis, improperly based on hingsight, and has also improperly gone outside the prior art to find the necessary motivation.

Based upon the foregoing, Appellants respectfully request reconsideration of the claims on the merits in light of the foregoing remarks, and allowance of the pending claims.

Respectfully submitted,



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Syngenta Biotechnology, Inc.
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Date: November 13, 2006

Claims Appendix

16. (Currently amended) A method of producing plant cells that accumulate carotenoids which cells are normally carotenoid-free said method comprising transforming plant material with an isolated DNA molecule comprising a nucleotide sequence which comprises:

(a) an expression cassette capable of directing production in said cells of a phytoene synthase derived from a plant; and

(b) an expression cassette capable of directing production in said cells of a phytoene desaturase derived from a bacteria; and
selecting transformed plant material that comprises the cells that accumulate carotenoids.

32. A method according to claim 16 wherein said phytoene desaturase is from the CrtI gene of *Erwinia uredovora*.

33. A method according to claim 16 wherein said phytoene desaturase is fused with a suitable plastid transit peptide.

34. A method according to claim 16 wherein said phytoene desaturase is expressed under the control of a tissue specific or constitutive promoter.

35. A method according to claim 34 wherein said phytoene desaturase is expressed under the control of a constitutive promoter.

36. A method according to claim 16 wherein said phytoene synthase is expressed under the control of a tissue specific promoter.

37. A method according to claim 36 wherein said phytoene synthase is derived from *Narcissus pseudonarcissus*.

38. A method according to claim 16 wherein said DNA further comprises a polynucleotide which provides for a selectable marker.

39. A method according to claim 16 wherein said plant material is transformed via an *Agrobacterium* which comprises said DNA.

40. A method according to claim 16 wherein said plant cell is a rice plant cell.

41. A method according to claim 16 wherein said cell is an endosperm cell.

42. A transformed plant cell obtainable by a method of claim 16.

43. A plant cell according to claim 42 which is a rice endosperm cell.

Evidence Appendix

In re application of:
Beyer, et al.
Serial Number: 09/914,913

The following are attached hereto:

Exhibit A: Interview Summary of telephonic interview between Michael E. Yates (Reg. No. 36,063), Attorney for Applicants/Appellants, and Examiner Kallis on August 11, 2006

Exhibit B: Auto-Reply Facsimile Transmission for Response to Final Office Action on August 15, 2006

Exhibit C: Response to Final Office Action, filed August 15, 2006

D1. The Rockefeller Foundation (1993) "Potential for Carotenoid Biosynthesis in Rice Endosperm". International Program on Rice Biotechnology; Workshop Report June 10-11.

D2. Burkhardt et al. (1996) "Genetic Engineering of Provitamin A biosynthesis in Rice Endosperm". Proceedings of the Third International Rice Genetics Symposium IRRI pp 818-820.

D3. Ye *et al.* (2000) "Engineering the Provitamin A (β-carotene) Biosynthetic Pathway into (Carotenoid-Free) Rice Endosperm". Science 287 pp 301-303.

D4. Bramley *et al.* (1997) Pure & Appl. Chem., Vol. 69, No. 10, pp.2159-2162.

D5. Potrykus (2001) "Golden Rice and Beyond". Plant Physiology 125 pp.1157-1161.

EXHIBIT A

Appellants wish to thank Examiner Kallis for his time and attention in connection with the telephonic interview held with the undersigned Attorney for Appellants on August 11, 2006. The following is intended to provide a brief summary of the interview.

Unfortunately, the Examiner had not received the fax of the draft response prior to the interview, but the interview proceeded with an initial discussion of the outstanding §102(b) rejection and Appellants' arguments in response (set forth in detail below). The Examiner noted the statements in D5 raised by Appellants in response to the D1 reference, but the Examiner also noted that reference D2 could support his position. Appellants' attorney disagreed, noting that D2 teaches the need to introduce all of the enzymes required for carotenoid production, not just the two of the claimed invention. The Examiner countered that it was his belief that at least one enzyme in the pathway is multi-functional and that it might be the case that use of that enzyme would be expected to accomplish more than one enzymatic reaction. Later the Examiner identified the reference as Misawa et al.

Examiner Kallis went on to argue that Burkhardt et al. (Plant J. 1997) (D6) and WO99/07867 A1 (D7) both tend to counter Appellants' position that, prior to the making of the present invention, the prior art reflected a low expectation of success in making plants capable of accumulating carotenoids. Appellants' attorney noted that the concluding paragraph of Burkhardt et al. is merely an indication of intended work and not at all a refelection of a reasonable expectation of success in arriving at the claimed invention. Appellants' attorney did not have reference D7 at hand and was unable to comment on this reference.

Appellants' attorney stated that the amendments to the claims set forth above would be submitted, and Examiner Kallis suggested that the amendments and remarks herein, along with this interview summary, be filed and put on the record. The Examiner indicated that he would futher consider the amendments in view of the accompanying remarks. The additional references relied upon by the Examiner in the interview have not been considered in the remarks below, since they were not cited in

the outstanding office action. Appellants respectfully request that the amendments be entered, since they at least put the case in better form for appeal, although Appellants submit that the amendments put the claims in condition for allowance.

Exhibit B

Auto-Reply Facsimile Transmission

TO: Fax Sender at 1+919+541+8689

Fax Information
Date Received: 8/15/2006 11:30:29 AM [Eastern Daylight Time]
Total Pages: 15 (including cover page)

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FROM: SYNGENTA PATENT DEPARTMENT (TUE) 8. 15' 06 (08:28/ST. 10:29/NO. 486; 4495[illegible] P 1

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Fax

To	United States Patent and Trademark Office Central Facsimile and Examiner Russell Kallis	Date:	August 15, 2006
Fax No:	571 273 8300 571 273 0798	Number of pages:	15
Concerning	**Response to Final Office Action for U.S. Serial No. 09/914,913**		

PAGE 1/15 * RCVD AT 8/15/2006 11:30:29 AM [Eastern Daylight Time] * SVR:USPTO-EFXRF-5/13 * DNIS:2738300 * CSID:1+919+541+8689 * DURATION (mm-ss):02-54

Exhibit C

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To	United States Patent and Trademark Office Central Facsimile and Examiner Russell Kallis	Date:	August 15, 2006
Fax No:	571 273 8300 571 273 0798	Number of pages:	15
Concerning	**Response to Final Office Action for U.S. Serial No. 09/914,913**		

Copy sent to C. Andrews
on 8/15/2006
by M. Hardy

MAH
Initials

Attorney Docket No. 60085USPCT
U.S. Serial No. 09/914,913

CERTIFICATE OF TRANSMISSION UNDER 37 C.F.R. § 1.8

I hereby certify that the following correspondence is being facsimile transmitted to the United States Patent and Trademark Office on August 15, 2006.

1) Response to Final Office Action (11 pages total)
2) Petition for Extension of Time
3) Credit Card Payment Form

Melissa Hardy
Name

Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Beyer, et al.	Art Unit: 1638
Serial Number: 09/914,913	Examiner: Russell Kallis
Filed: December 17, 2001	Confirmation No.: 5922
For: Method for Improving the Agronomics and Nutritional Value of Plants	Atty Docket: 60085 USPCT

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO FINAL OFFICE ACTION

This paper is filed in response to the Final Office Action mailed on March 28, 2006 which has a shortened statutory time set to expire on June 28, 2006. A two month extension to August 28, 2006 is hereby requested pursuant to 37 CFR §1.136(a). Reconsideration in light of the accompanying remarks, with early allowance of the pending claims, is respectfully requested.

Please charge Applicants' credit card in the amount of $450.00 for payment of the extension fee. Enclosed is a Credit Card Payment Form for fee purposes. The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment, to Deposit Account No. 50-1744 in the name of Syngenta Biotechnology, Inc.

Amendments to the Claims

1 - 15. (Cancelled)

16. (Currently amended) A method of producing plant cells that accumulate ~~carotenoids~~ β-carotene which cells are normally carotenoid-free said method comprising transforming plant material with an isolated DNA molecule comprising a nucleotide sequence which comprises:

(a) an expression cassette capable of directing production in said cells of a phytoene synthase derived from a plant; and

(b) an expression cassette capable of directing production in said cells of a phytoene desaturase derived from a bacteria; and
selecting transformed plant material that comprises the cells that accumulate ~~carotenoids~~ β-carotene.

17 - 31. (Cancelled)

32. (Previously presented) A method according to claim 16 wherein said phytoene desaturase is from the CrtI gene of *Erwinia uredovora.*

33. (Previously presented) A method according to claim 16 wherein said phytoene desaturase is fused with a suitable plastid transit peptide.

34. (Previously presented) A method according to claim 16 wherein said phytoene desaturase is expressed under the control of a tissue specific or constitutive promoter.

35. (Previously presented) A method according to claim 34 wherein said phytoene desaturase is expressed under the control of a constitutive promoter.

36. (Previously presented) A method according to claim 16 wherein said phytoene synthase is expressed under the control of a tissue specific promoter.

37. (Previously presented) A method according to claim 36 wherein said phytoene synthase is derived from *Narcissus pseudonarcissus*.

38. (Previously presented) A method according to claim 16 wherein said DNA further comprises a polynucleotide which provides for a selectable marker.

39. (Previously presented) A method according to claim 16 wherein said plant material is transformed via an *Agrobacterium* which comprises said DNA.

40. (Previously presented) A method according to claim 16 wherein said plant cell is a rice plant cell.

41. (Previously presented) A method according to claim 16 wherein said cell is an endosperm cell.

42. (Previously presented) A transformed plant cell obtainable by a method of claim 16.

43. (Previously presented) A plant cell according to claim 42 which is a rice endosperm cell.

Claims 44 – 59 (Cancelled).

60. (New) A method of producing rice plants that accumulate β-carotene in endosperm cells, said method comprising transforming plant material with an isolated DNA molecule comprising a nucleotide sequence which comprises:
(a) an expression cassette capable of directing production in said cells of a phytoene synthase derived from a plant; and

(b) an expression cassette capable of directing production in said cells of a phytoene desaturase derived from a bacteria;
and selecting transformed plant material that comprises the cells that accumulate β-carotene.

REMARKS

The present invention provides methods for making transformed plants, plant cells, seeds, tissues etc. capable of expressing enzymes of the carotenoid biosynthesis pathway essential to the accumulation of carotenes and/or xanthophylls of interest. The present invention further provides DNA molecules designed to be suitable for producing such plants and plant parts. The currently pending claims relate to the method for making such plants, and to plants made using the method. Claims 1-15, 17-31, and 44-59 are cancelled. Claims 16 and 32-43 are pending and under final rejection. The references cited against the pending claims in the outstanding office action are listed here:

D1. The Rockefeller Foundation (1993) "Potential for Carotenoid Biosynthesis in Rice Endosperm". International Program on Rice Biotechnology; Workshop Report June 10-11.

D2. Burkhardt et al. (1996) "Genetic Engineering of Provitamin A biosynthesis in Rice Endosperm". Proceedings of the Third International Rice Genetics Symposium IRRI pp 818-820.

D3. Ye *et al.* (2000) "Engineering the Provitamin A (β-carotene) Biosynthetic Pathway into (Carotenoid-Free) Rice Endosperm". Science 287 pp 301-303.

D4. Bramley *et al.* (1997) Pure & Appl. Chem., Vol. 69, No. 10, pp.2159-2162.

In addition, Applicants are submitting herewith, for the Examiner's consideration in connection with the remarks set out below, the following reference:

D5. Potrykus (2001) "Golden Rice and Beyond". Plant Physiology 125 pp.1157-1161.

Interview Summary

Applicants wish to thank Examiner Kallis for his time and attention in connection with the telephonic interview held with the undersigned Attorney for

Applicants on August 11, 2006. The following is intended to provide a brief summary of the interview.

Unfortunately, the Examiner had not received the fax of the draft response prior to the interview, but the interview proceeded with an initial discussion of the outstanding §102(b) rejection and Applicants' arguments in response (set forth in detail below). The Examiner noted the statements in D5 raised by Applicants in response to the D1 reference, but the Examiner also noted that reference D2 could support his position. Applicants' attorney disagreed, noting that D2 teaches the need to introduce all of the enzymes required for carotenoid production, not just the two of the claimed invention. The Examiner countered that it was his belief that at least one enzyme in the pathway is multi-functional and that it might be the case that use of that enzyme would be expected to accomplish more than one enzymatic reaction. Later the Examiner identified the reference as Misawa et al.

Examiner Kallis went on to argue that Burkhardt et al. (Plant J. 1997) (D6) and WO99/07867 A1 (D7) both tend to counter Applicants' position that, prior to the making of the present invention, the prior art reflected a low expectation of success in making plants capable of accumulating carotenoids. Applicants' attorney noted that the concluding paragraph of Burkhardt et al. is merely an indication of intended work and not at all a refelection of a reasonable expectation of success in arriving at the claimed invention. Applicants' attorney did not have reference D7 at hand and was unable to comment on this reference.

Applicants' attorney stated that the amendments to the claims set forth above would be submitted, and Examiner Kallis suggested that the amendments and remarks herein, along with this interview summary, be filed and put on the record. The Examiner indicated that he would futher consider the amendments in view of the accompanying remarks. The additional references relied upon by the Examiner in the interview have not been considered in the remarks below, since they were not cited in the outstanding office action. Applicants respectfully request that the amendments be entered, since they at least put the case in better form for appeal, although Applicants submit that the amendments put the claims in condition for allowance.

Claim Rejections Under 35 U.S.C. §102

Claims 16 and 32-43 remain rejected under 35 U.S.C. §102(b) as "being anticipated under [D1]". The Examiner disputes our previous assertion that D1 represents early stage discussions and experiments by experts in the field in assessing the potential for carotenoid production in rice. The Examiner highlights that D1 recites "Amidst optimism that the goal of accumulating nutritionally significant levels of carotenoid synthesis could be achieved the meeting was adjourned".

Two specific "strategies" for providing carotenoid accumulation in the rice endosperm are outlined in D1: (1) a "non-green option" (comprising a bacterial phytoene synthase and a bacterial phytoene desaturase); and (2) a "mixed option" (comprising a plant phytoene synthase and a plant phytoene desaturase). Although D1 does discuss both plant and bacterial genes, it does not specifically disclose a method comprising a combination of a plant phytoene synthase and a bacterial phytoene desaturase. The Examiner has combined separate and distinct elements disclosed in D1 in order to arrive at the present invention, but it is well settled in the law that for a reference to anticipate under 35 U.S.C.§102, every element of the claimed invention must be identically shown in a single reference, and the elements must be arranged as in the claim under review.

Applicants realize that it is also well settled that the legal premise just set out is not "ipsissmis verbis" (is not "expressly...in words"), so that D1 could be considered anticipatory if a person of ordinary skill in the art would understand the reference as disclosing all elements, in proper combination or arrangement, based on their own knowledge and the state of the art. However, in this regard Applicants respectfully draw the Examiner's attention to document D5, submitted herewith, which provides a history of "Golden Rice" (to which the present invention relates). The author of D5 is Professor Ingo Potrykus, who is named as a co-inventor in respect of the present application and who was present at the workshop summarised in D1.

On page 1158 of D5 it is stated "the verdict of this initial session [the Rockefeller workshop] was that such a project had a low probability of success, but

that it was worth trying because of its high potential benefit" (emphasis added). Furthermore, it is stated "There are hundreds of scientific reasons why the introduction and coordinated function of these enzymes would not be expected to work. Those with the necessary scientific knowledge were right in not believing in the experiment" (emphasis supplied). Indeed, even reference D1 concludes, *inter alia*, that "it is likely that beta-carotene accumulation in the rice endosperm will require the introduction of the complete set of genes coding for phytoene synthase onwards" (Appendix D). In addition, D5 highlights the scepticism that existed at the time of D1 concerning the feasibility of successfully engineering an entire biosynthetic pathway into rice. The expectation of success in achieving the desired goal was thus extremely low.

However, contrary to the suggestion of D1, the methods of the present invention do not require a "complete set" of carotenoid biosynthetic genes in order to produce β-carotene in the rice endosperm. Indeed, it came as a surprise that β-carotene, rather than lycopene, accumulation was achieved via the use of only a plant phytoene synthase and a bacterial phytoene desaturase (as evidenced in reference D3, which is discussed further below) in the absence of engineering into the rice plant a lycopene cyclase. Thus, D1 can be seen to teach away from the present invention. Although D1 does recite a plant phytoene synthase and a bacterial phytoene desaturase, it contains no suggestion or motivation to combine these distinct features in order to provide a method to accumulate β-carotene in the rice endosperm.

Thus, although D1 suggests that there may have been "optimism" that significant levels of carotenoid synthesis could be achieved – there actually existed significant doubt among those of skill in the art (as discussed in D5) that a result would actually be achievable. At best, D1 can be seen as an invitation to carry out further research – the reality being that the methods of the present invention for providing β-carotene accumulation in the rice endosperm (the language adopted in new claim 60) actually took many years to achieve. This, combined with the fact that D1 does not disclose the claimed combination of a plant phytoene synthase with a bacterial phytoene desaturase, leads to the conclusion that D1 is not anticipatory under 35 U.S.C. §102(b).

The Examiner has also relied on reference D3, Ye et al., in arriving at the conclusion that "The Rockefeller reference teaches inherently, a method of producing plant cells that accumulate carotenoid by transformatino with a gene from daffodil...encoding a phyotene synthase and a gene from daffodil...encoding a phyotene desaturase or by transformation with a gene from daffodil encoding a phytoene synthase and a bacterial *crtI* gene...encoding a phytoene desaturase and plant cells and plants transformed thereby...." However, the foreign priority document to which the present application claims priority was filed on March 5, 1999, and D3 was published in the 14 January 2000 issue of Science. Furthermore, D3 represents the work of Beyer and Potrykus as disclosed in the present application, and thus could not have appeared before the making of the claimed invention. In any event, the publication of D3 occurred well after the priority date, and less than one year from the §371 international filing date (March 3, 2000), and therefore D3 is not available as a reference under either 35 U.S.C.§102(a) or (b).

Therefore, for the foregoing reasons it is respectfully submitted that the subject matter of the presently submitted claims is in fact novel over D1, and Applicants respectfully request that this rejection be withdrawn.

Claim Rejections Under U.S.C §103

Claims 16 and 32-43 are rejected under 35 U.S.C.§103 as allegedly being obvious over D2 and D4. The Examiner suggests that, in view of D2 and D4, "one would have had reasonable expectation of success in transforming a rice plant with a plant phytoene synthase and a bacterial phytoene desaturase; and in producing provitamin A or beta carotene in the endosperm of rice". Respectfully, Applicants strongly disagree with this assertion, for the following reasons.

As discussed above with regard to D1, D2 suggests (page 819) that four enzymes are necessary for β-carotene biosynthesis in the rice endosperm (phytoene synthase, phytoene desaturase, ζ-carotene desaturase and lycopene cyclase). Thus D2, like D1, teaches away from the claimed method for reasons already stated above.

D2 also discloses a transgenic rice plant purported to comprise a plant (daffodil) phytoene synthase and a plant (daffodil) phytoene desaturase. It is reported that the transgenic rice plant accumulated a single carotenoid (phytoene) but not ζ-carotene, the product of phytoene desaturase (page 820).

The work reported in D2 was important since it indicated that was possible to produce phytoene in the rice endosperm (indeed this was considered to be the "first breakthrough" in the development of Golden Rice - D5 page1158). However, D2 does not solve the problem of the production of carotenoids other than phytoene in the rice endosperm – since the phytoene desaturase that was introduced did not result in the accumulation of ζ-carotene as expected. Furthermore, D2 does not teach, or even suggest, a method by which β-carotene can be produced in the rice endosperm. There is certainly no suggestion in D2 that β-carotene can be produced in the rice endosperm by replacing the plant phytoene desaturase with a bacterial phytoene desaturase. Indeed, the skilled person would understand from D1 that the use of bacterial carotenoid gene(s) to produce carotenoids in plants is not ideal – since, while they appear to be functional, they can result in non-viable plants (D1 - page 3).

Bramley *et al.* (D4) disclose, *inter alia*, the use of a bacterial phytoene desaturase gene (*crt* I) to increase carotenoid levels in tissues (tomato fruit) which already have the capacity to synthesis high levels of carotenoids. Thus the skilled person would appreciate that, in the methods taught by Bramley, all of the biosynthetic machinery required to produce carotenoids are already present in the tissues transformed with the *crt*I gene. D4 is thus not concerned with the problem addressed by present invention – that is the accumulation of carotenoids in tissues which are normally carotenoid free.

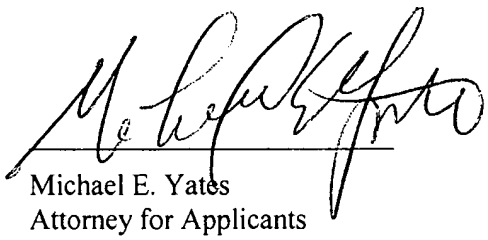
There is no suggestion in either D2 or D4 that β-carotene accumulation could be achieved in the rice endosperm by the use of a plant phytoene synthase and a bacterial phytoene desaturase (e.g *crt* I) alone. Although it is possible to combine the teachings of D2 and D4 and arrive at the present invention there is absolutely no motivation to do so in the absence of the present invention. In doing so, it is submitted that the Examiner has made an *ex-post facto* analysis based on hingsight. Therefore,

Applicants respectfully submit that the present rejection has been addressed, and should be withdrawn.

CONCLUSION

Applicants respectfully request reconsideration of the claims on the merits in light of the foregoing remarks, and allowance of the pending claims. Applicants are also filing herewith a notice of appeal. Should the Examiner consider a telephone call to the attorney for Applicants to be helpful in progressing the case to allowance one is earnestly requested.

Respectfully submitted,



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Date: August 15, 2006

D1

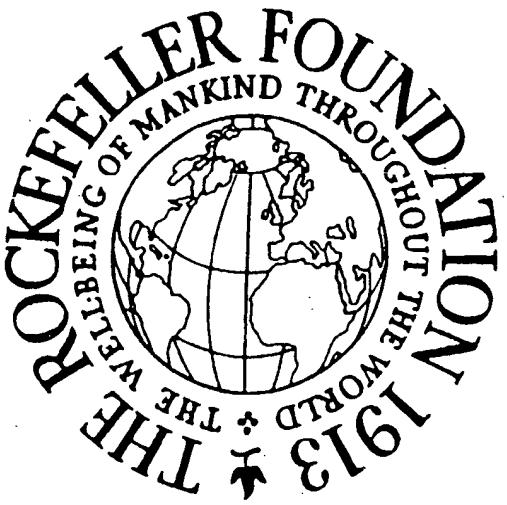
INTERNATIONAL PROGRAM

ON

RICE BIOTECHNOLOGY

WORKSHOP REPORT

POTENTIAL FOR CAROTENOID BIOSYNTHESIS IN RICE ENDOSPERM



JUNE 1993

Summary Report

Rockefeller Foundation Workshop

on

Potential for Carotenoid Biosynthesis in Rice Endosperm

held

June 10-11, 1993

at

The Rockefeller Foundation
1133 Avenue of the Americas
New York, NY 10036 USA

Rapporteur:

Elizabeth Evans
Warren Weaver Fellow
Agricultural Sciences

Rockefeller Foundation Workshop
on
The Potential for Carotenoid Biosynthesis in Rice Endosperm

June 10-11, 1993

Summary Report

A group of carotenoid biochemists and geneticists, and plant molecular biologists was assembled in order to evaluate the feasibility of alternatives for introducing or activating the carotenoid biosynthesis pathway in rice endosperm. Dr. Toenniessen began the meeting by explaining that carotenoids (provitamin A) in endosperm had been identified as a desirable trait to introduce into rice, since none occur naturally, and rice-consuming populations (particularly children) suffer substantial morbidity and mortality due to vitamin A deficiencies. RF has made two grants to explore the components of the carotenoid pathway in maize, since the yellow maize endosperm produces some carotenoids, and a small grant was also made to explore the status of carotenoid precursors in the rice endosperm.

Dr. Olson outlined the functions of vitamin A in mammals and the FAO/WHO recommendations for provitamin A levels in the diet. To provide the minimum requirements of relevant carotenoids (assuming rice as the sole dietary source), 1-2 micrograms beta-carotene per gram uncooked rice would need to be synthesized in the endosperm, roughly 2 to 4 times that produced by maize endosperm, and enough to turn the rice noticeably but not dark yellow. Beta-carotene accounts for only about 10% of the

pigmentation of yellow maize with the rest being zeaxanthin and other pigments formed after beta-carotene in the pathway (see Appendix C). Cooking practice could affect the longevity of carotenoids, and they must be consumed with sufficient oils to facilitate absorption. Carotenoids at this level may change the physical and cultural acceptability of the rice.

Dr. Beyer described the compartmentalization and topology of the carotenoid pathway in higher plants, and discussed the possible competition for substrates and requirement for redox cofactors in rice. Dr. Britton summarized the biochemical analysis of rice endosperm which detected the precursors FPP and GGPP in small amounts (appendix D). The limitations of this small study were noted, particularly the single time point. Dr. Wurtzel outlined a variety of maize mutants that may provide an analogy to the situation in rice. Lethal recessive viviparous mutants are blocked somewhere between phytoene and ABA (plant hormone that suppresses germination), dominant non-viviparous mutants appear blocked between MVA and phytoene. Dr. Camara reported on the cloning of the GGPP synthase gene in pepper, and the preparation of antibodies against pepper phytoene synthase and phytoene desaturase that have been used to follow the production and transport of enzymes into chromoplasts.

Dr. Potrykus outlined progress in developing stable and transient transformation systems for rice. He suggested that microtargeting liquid droplets into rice tissue would be the best

way to test DNA constructs, enzymes, precursors, etc. in a transient assay. Substrates as well as genes can be delivered. Endosperm cultures might also be useful. Dr. Bogorad discussed the hypothetical nature of the block to carotenoid synthesis in rice endosperm suggesting that there may be lack of substrates, lack of genes (unlikely since the leaves produce carotenoids), improper compartments/membranes, feedback inhibition, or a block to the transcription, translational or post-translation modification of genes in the pathway. The two approaches to overcoming the block would be 1) to turn on the existing pathway, or 2) introduce exogenous genes encoding all or part of the pathway.

Dr. Ausich outlined a project at Amoco where cloned genes for the whole pathway from *Erwinia herbicola* were introduced into tobacco and yeast. In yeast, each gene in the pathway was introduced sequentially, with the appropriate next product produced at each step. In tobacco, simple 35S promoters and rubisco leader sequences were employed. In either case, the cells turned orange due to the production of carotenoids. Although the orange tobacco seedlings died, these stunning results suggested that bacterial genes can work in plants, and may not have stringent requirements for targeting and coordination of enzymes in plastid membranes that have been observed in higher plants. Dr. Misawa described the introduction of the *E. uredovora* phytoene desaturase gene into tobacco which produced functional resistance to norflurazon, an inhibitor of the plant enzyme. Dr. Scolnik

outlined the use of RT-PCR to study developmental expression of enzyme mRNAs in tomato, and suggested that the same could easily be done for rice. Dr. Hirschberg noted that sequential addition of cyanobacterial genes to *E. coli* resulted in the introduction of discrete steps of the pathway, suggesting again that the enzymes can operate independently, outside of a complex.

Dr. Armstrong summarized the cloned genes available from bacteria and plants, and outlined options for introducing all or part of the pathway into rice (see appendix E), assuming that FPP is present and available. The pros and cons of introducing higher plant or non-plant genes as cDNAs or genomic clones were debated. The *Erwinia* genes emerged as the most complete and well studied set now available. Issues to be determined include the number of sequences to add, promoter choice (endosperm specific), codon usage, targeting (cytosol or plastid), possible incompatibility of enzymes from different organisms, and the stereochemistry of the intermediates. The problem of trapping enough active provitamin A carotenoids (carotenes and cryptoxanthins) rather than running through to zeaxanthin or ABA was discussed. The potential disruption of other isoprenoid pathways was also noted.

Dr. Wurtzel stressed the need to understand the basic biology of the system in order to evaluate the experiments and options. Dr. Hearst described carotenoid mutants of *Erwinia*, and stressed the need for good assays to detect the different products of the pathway. Dr. Burr raised the possibility that regulatory gene(s)

rather than structural gene(s) may be the problem in rice endosperm, and presented a strategy for identifying and locating such genes. The existence of natural potato lines that are yellow (presumably in the amyloplast) and carrots that are white (wild type) raised hopes that switching on and off the pathway was not an impossible goal. The existence of albino and viviparous rice mutants was noted.

Dr. Quatrano summarized the points of discussion from the meeting and outlined a two-pronged experimental plan to address the crucial questions about the blocked pathway in rice endosperm, and explore the possibly introducing the pathway by genetic engineering. He noted that a prerequisite for the project is the optimization of the transient expression system for rice endosperm at early stages of its development.

EXPERIMENTAL PLAN

A. Identification of the Biochemical Lesion in Rice Endosperm

Experiment 1: Add the lycopene cyclase inhibitor CPTA to endosperm and see if lycopene is accumulated. This provides a quick way of determining if rice endosperm contains enzymes for the first portion of the pathway up to lycopene.

Experiment 2: Use antibodies to assess the presence or absence of enzymes for the pathway in rice endosperm. If Dr. Camara's antibodies against the dicot proteins can detect monocot enzymes this can readily be done. If not, rice specific antibodies will need to be developed.

Experiment 3: Assess the status of the pathway at the level of isoprenoid precursors and expression of genes for the enzymes (using RT-PCR) in a complete developmental time course comparing rice and maize endosperm. This should be complemented by a study of the time course and localization of carotenoid deposition in the maize amyloplast during development.

Part B: Introduction of the Pathway by Genetic Engineering

Experiment 1: Using microtarteting and transient expression, attempt to complement the y1 allele in maize using the *Erwinia* phytoene synthase gene with an appropriate promoter and transit peptide. This would determine if genes expressed transiently can function in an existing endosperm pathway. Similar positive controls for other gene constructs could be developed using appropriate maize mutants.

Experiment 2: Attempt to introduce gene(s) for the pathway (for example, *Erwinia* genes driven by the 35S promoter or endosperm specific rice promoters) into rice endosperm using the transient system.

Experiment 3: Use the conserved portions of the available plant gene sequences to develop primers in order to clone the rice genes.

Amidst optimism that the goal of accumulating nutritionally significant levels of carotenoid synthesis in rice endosperm could be obtained, the meeting was adjourned.

Appendix A

Rockefeller Foundation Workshop
on
Potential for Carotenoid Biosynthesis in Rice Endosperm

Agenda

Thursday, June 10 - Chair - Ralph Quatrano

9:00 AM Welcome and introductions
Purpose of the workshop - Gary Toenniessen
Biological action of carotenoids
and levels required - Jim Olson

10:00 Carotenoid biosynthesis in plants
Requirements - Peter Beyer
Enzymes, precursors and
blockages in rice endosperm - George Britton
Assembly and subcellular
localization of carotenoid pathway - Bilal Camara
Genetics of carotenoid pathway
in maize endosperm - Eleanor Wurtzel

12:00 Status of rice genetic engineering
Rice transformation - Ingo Potrykus
Tissue specific expression
and site directed delivery - Lawrence Bogorad

1:00 PM Lunch Break

2:00 Relevant experience to date with plants and other organisms engineered for modified carotenoid biosynthesis
- Rodney Ausich
- Norihiko Misawa
- Pablo Scolnik
- Joseph Hirschberg

5:00 Adjourn

Friday, June 11 - Chair - Gary Toenniessen

9:00 AM	Options for introducing genes for the pathway - Gregory Armstrong - Eleanor Wurtzel - John Hearst
10:30	Options for activating the pathway in rice endosperm - Ben Burr
11:00	Recap of findings/conclusions - Ralph Quatrano (lead discussant) Areas of agreement Areas of disagreement How realistic is the objective?
12:30 PM	Lunch
1:30	Strengths and weaknesses of alternative strategies for achieving goals - Ben Burr (lead discussant)
2:30	Recommend next steps
4:00	Adjourn

Appendix B

Rockefeller Foundation Workshop
on
Potential for Carotenoid Biosynthesis in Rice Endosperm

List of Participants

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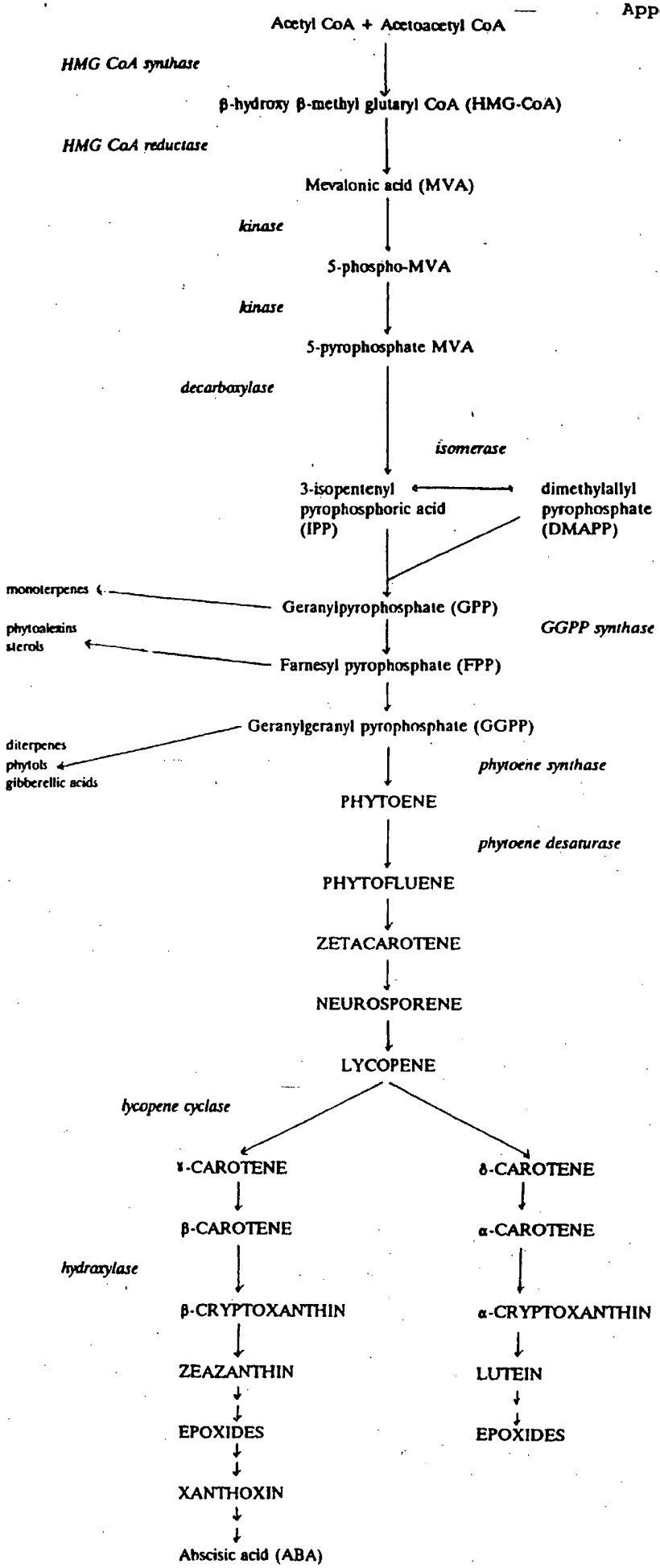

Acetyl CoA + Acetoacetyl CoA
HMG CoA synthase
β-hydroxy β-methyl glutaryl CoA (HMG-CoA)
HMG CoA reductase
Mevalonic acid (MVA)
kinase
5-phospho-MVA
kinase
5-pyrophosphate MVA
decarboxylase
isomerase
3-isopentenyl pyrophosphoric acid (IPP)
dimethylallyl pyrophosphate (DMAPP)
monoterpenes
Geranylpyrophosphate (GPP)
GGPP synthase
phytoalexins
sterols
Farnesyl pyrophosphate (FPP)
Geranylgeranyl pyrophosphate (GGPP)
diterpenes
phytols
gibberellic acids
phytoene synthase
PHYTOENE
phytoene desaturase
PHYTOFLUENE
ZETACAROTENE
NEUROSPORENE
LYCOPENE
lycopene cyclase
ɣ-CAROTENE
δ-CAROTENE
β-CAROTENE
α-CAROTENE
hydroxylase
β-CRYPTOXANTHIN
α-CRYPTOXANTHIN
ZEAZANTHIN
LUTEIN
EPOXIDES
EPOXIDES
XANTHOXIN
Abscisic acid (ABA)

Appendix D

SUMMARY

The Ability of Rice Endosperm to Make Isoprenoid (Carotenoid) Precursors

Results

1. In the varieties of rice examined, no trace of any carotenoid or C40 carotenoid precursor was detected in the endosperm.

2. Some residual chloroplast carotenoids, mainly beta-carotene and lutein, were present in the seedcoat of cultivated varieties and of 'wild rice'.

3. Other isoprenoid compounds (e.g. sterols, tocopherol) were present in extracts of mature endosperm, though not in large quantities.

4. Incubation of mature endosperm with isoprenoid precursors, mainly mevalonate, gave only low incorporation.

5. Incorporation by immature endosperm was greater, though still not large and radioactivity was demonstrated in non-carotenoid products. No radioactivity was recovered in phytoene or coloured carotenoids.

6. With 'young' developing seeds, incorporation into prenyl diphosphates, largely the C15 farensyl diphosphate, but a low level also in the C20 geranylgeranyl diphophate could be demonstrated.

Conclusions

1. The ability to biosynthesize carotenoids or their precursors with the C40 skeleton, especially phytoene, is lacking from rice endosperm, though chloroplast-containing tissues, including the seed coat, do have this ability.

2. The developing seeds are able to biosynthesize other isoprenoid compounds. Prenyl transferase activity up to C15 (farnesyl diphosphate) and C20 (geranylgeranyl diphosphate) is present, though at a comparatively low level. Detailed study during the course of development might well reveal a short peak in GGDP synthesis *en route* to gibberellins.

3. It is likely that beta-carotene accumulation in rice endosperm will require the introduction of the complete set of genes coding for phytoene synthase onwards. These genes are present and active during chloroplast development in green parts of the plant but it is not known how they could be 'switched on' in the endosperm tissue.

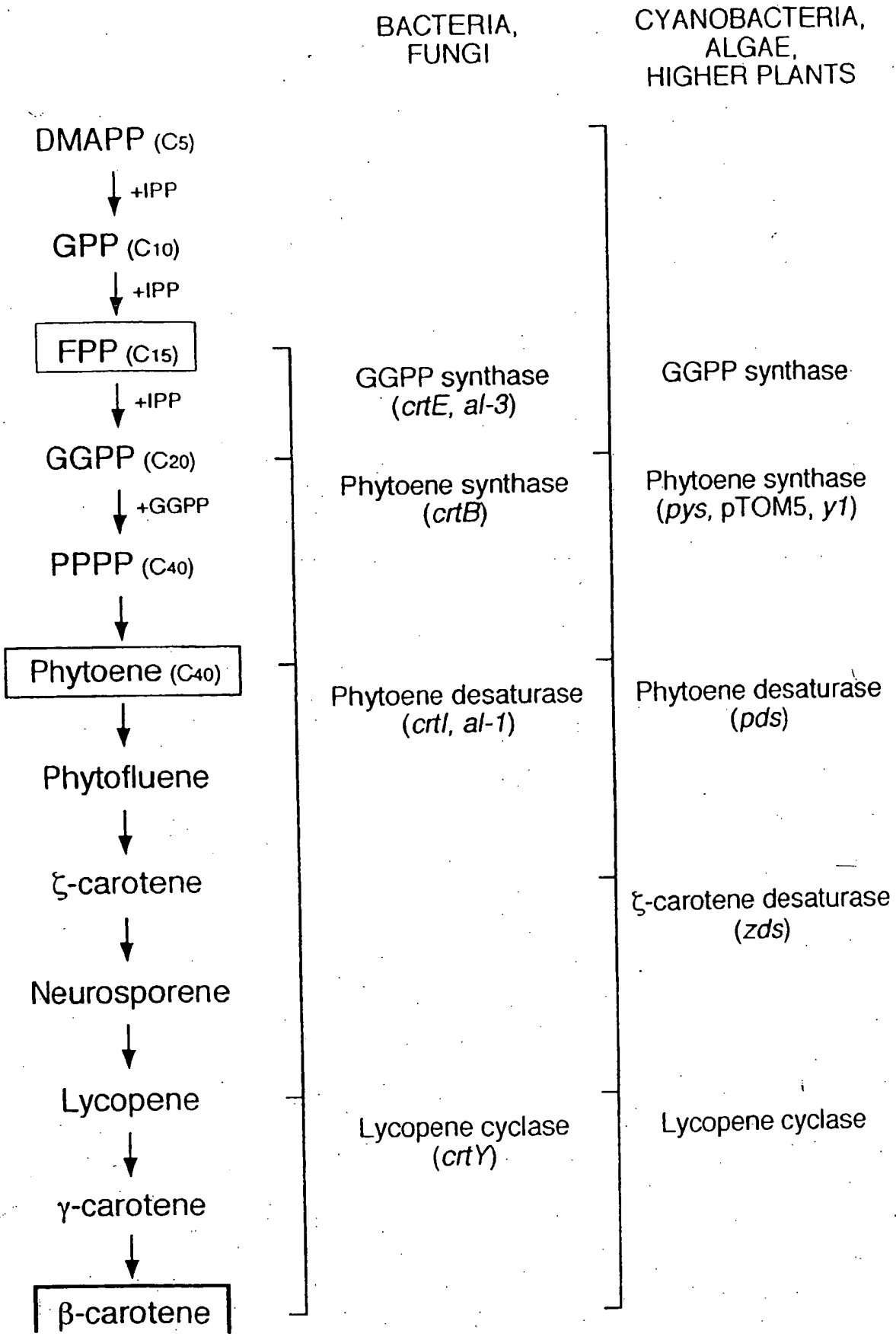
4. All tissues, including endosperm, have the fundamental ability to carry out the early stages of isoprenoid biosynthesis, including the formation of FDP and GGDP *en route* to other isoprenoid compounds. Increased activity of the prenyl transferase enzymes may be necessary, however, if an appreciable level of beta-carotene is to be produced.

George Britton
Department of Biochemistry
University of Liverpool

15th December 1992

Appendix E
Prepared by G. Armstrong

GENERAL SCHEME FOR β-CAROTENE SYNTHESIS



Genes and cDNAs available from "non-green" organisms

	GGPP synthase	Phytoene synthase	Phytoene desaturase*	Lycopene cyclase
Rhodobacter capsulatus	*crtE* (g)[2]	*crtB* (g)[2]	*crtI***(g)[2,3]	
Erwinia herbicola	*crtE* (g)[1]	*crtB* (g)[1]	*crtI* (g)[1]	*crtY* (g)[12]
Erwinia uredovora	*crtE* (g)[15]	*crtB* (g)[15]	*crtI* (g)[15]	*crtY* (g)[15]
Neurospora crassa	*al-3* (c,g)[8]	*al-2* ?	*al-1* (c,g)[19]	

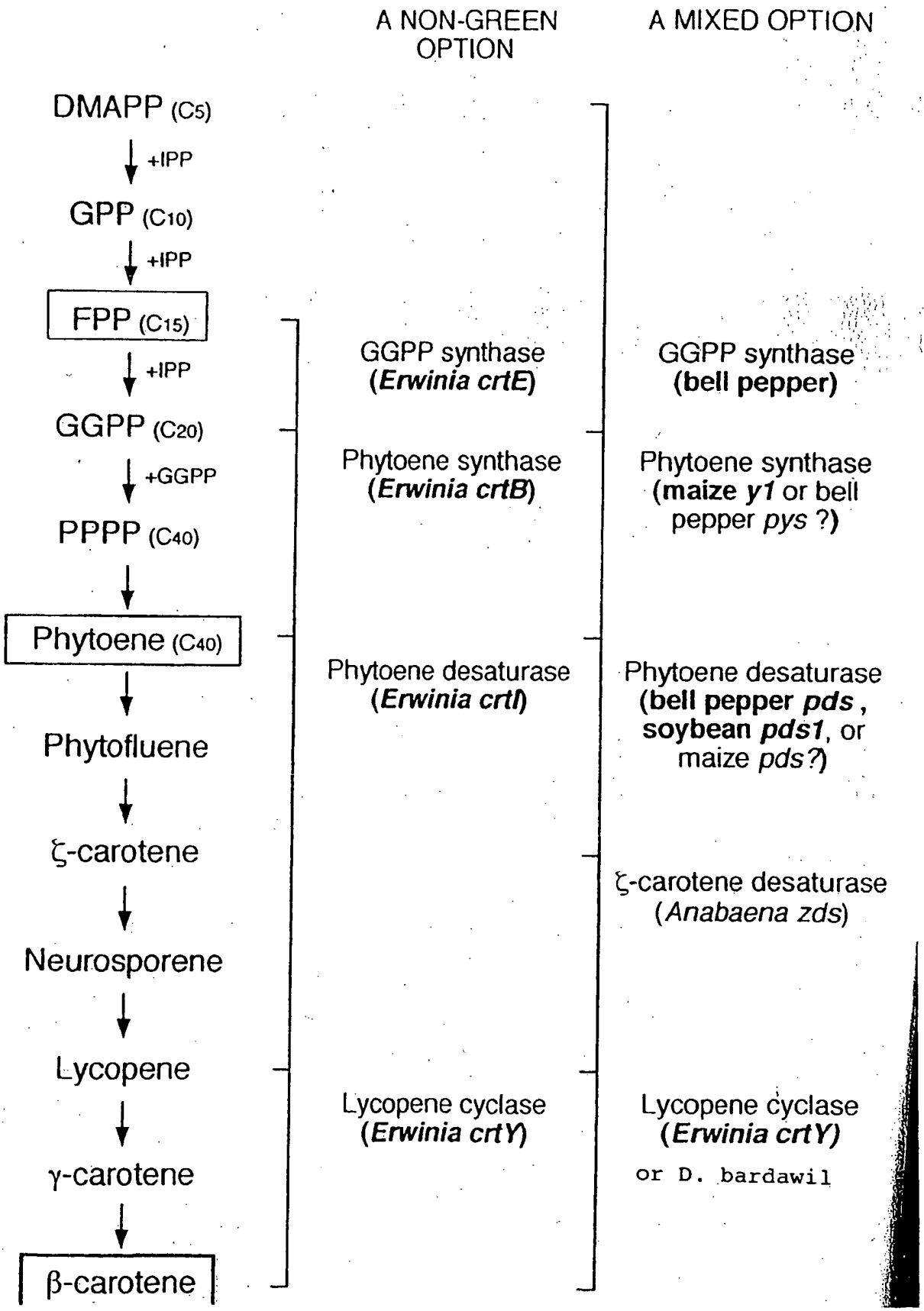
*phytoene to lycopene; **phytoene to neurosporene

Genes and cDNAs available from "green" organisms

	GGPP synthase	Phytoene synthase	Phytoene desaturase*	ζ-carotene desaturase**	Lycopene cyclase
Synechocystis PCC 6803			*pds* (g)[10]		
Synechococcus PCC 7942		*pys* (g)[9]	*pds* (g)[16]		
Anabaena PCC 7120				*zds*** (g)[14]	
Dunaliella bardawil			*pds* (c)[16]		lycopene cyclase
Tomato		pTOM5(c,g)[17,18] *Psy1* (c)[4]			
Soybean			*pds1* (c)[5]		
Bell pepper	GGPPS(c)[13]	*pys* ?	*pds* (c)[11]		
Maize		*y1* (c,g)[6,7]	*pds* ?		

*phytoene to ζ-carotene; **ζ-carotene to lycopene; ***not sequenced

STRATEGIES FOR OBTAINING β-CAROTENE SYNTHESIS IN ENDOSPERM



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D2

Rice Genetics III

Proceedings of the

G. S. Khush ed.

Manila, Philippines • 16-20 Oct 1995

1996

IRRI

International Rice Research Institute

P.O. Box 933, Manila 1099, Philippines

Genetic engineering of provitamin A biosynthesis in rice endosperm

P.K. Burkhardt, P. Beyer, R. Terada, A. Klöti, J. Wünn, J. v. Lintig, G.A. Armstrong, and I. Potrykus

Rice is characterized by a complete absence of provitamin A in its endosperm. Provitamin A, in the form of ß-carotene or structurally related compounds, is the essential precursor for the production of vitamin A in animals and man. Vitamin A deficiency causes 1.3-2.5 million deaths annually among young children worldwide. Furthermore, it is estimated that some 5 million preschool children in Southeast Asia become at least partially blind. Here, we present experiments that aim to initiate the first steps of provitamin A biosynthesis in rice endosperm through genetic engineering. Investigation of the biochemical status of immature rice endosperm has revealed the presence of the isoprenoid precursors necessary for carotenoid biosynthesis but no further hydrocarbon intermediates required to produce ß-carotene. Subsequently, cDNAs encoding the first two of four specific enzymes of this pathway have been transformed into a japonica rice variety and accumulation of the intermediate phytoene could be demonstrated.

According to statistics of the United Nations International Children's Emergency Fund, more than 134 million children worldwide are estimated to be suffering from vitamin A deficiency (Humphrey et al 1992). Improved vitamin A nutrition would be expected to prevent approximately 1-2 million deaths annually among children aged 1-4 yr. An additional 0.25-0.5 million deaths could be avoided if improved vitamin A nutrition could be achieved during late childhood. Improved vitamin A nutrition alone could therefore prevent 1.3-2.5 million out of nearly 8 million late infancy and preschool age children deaths that occur each year in the highest risk countries (West et al 1989).

Rice is the major food staple for most people in Southeast Asia. Its endosperm, however, completely lacks ß-carotene or structurally related compounds that can serve as essential provitamins for the production of vitamin A and retinal and retinoic acid in animals and man. The milled rice kernel consists exclusively of the endosperm

because the embryo and the aleurone layer have been removed during processing. Thus, many children worldwide, particularly in large areas of Southeast Asia, suffer from a variety of mild to severe health problems resulting from vitamin A deficiency.

This project aims to initiate carotenoid biosynthesis in the rice endosperm tissue to increase the daily vitamin A uptake of potentially vitamin A-deficient people who rely predominantly on rice as a food source.

For maize and sorghum, it is known that endosperm cells of cereals can produce and accumulate carotenoids (Buckner 1993). Furthermore, the starch storage tissues of potato and cassava (Penteado and Almeida 1988) accumulate carotenoids in considerable amounts.

To provide the minimum requirements of relevant carotenoids to young infants, and assuming that rice is the sole dietary source, 1-2 mg ß-carotene g^{-1} of uncooked rice would be needed in rice endosperm (The Rockefeller Foundation 1993). This is roughly 1/4-1/2 of the amount produced in the endosperm of certain maize varieties, and enough to turn the rice noticeably, though not intensely, yellow.

The carotenoid biosynthesis pathway is a branch of the general isoprenoid pathway. The four enzymes that are necessary for ß-carotene biosynthesis in this branch are phytoene synthase, phytoene desaturase, ζ-carotene desaturase, and lycopene cyclase. The genes for these enzymes are available from higher plants (Ray et al 1987, Bartley et al 1991, Fray and Grierson 1993), purple photosynthetic bacteria (Armstrong et al 1989), cyanobacteria (Linden et al 1994), and nonphotosynthetic bacteria (Misawa et al 1990).

Initially, we analyzed the biochemical status of immature rice endosperm. Incubation assays with radiolabeled precursors of general isoprenoid biosynthesis demonstrated the presence of geranyl geranyl pyrophosphate (GGPP), the substrate for the first specific enzyme of carotenoid biosynthesis, phytoene synthase.

Our strategy is to produce transgenic rice varieties that contain either single hetorologous carotenoid biosynthesis genes or several genes in combination. For this approach, we have chosen sequences encoding the enzymes phytoene synthase and phytoene desaturase from daffodil (*Narcissus pseudonarcissus*), which is a monocot plant like rice. Daffodil phytoene synthase and phytoene desaturase (P. Beyer 1995, University of Freiburg, Germany, unpubl. data) cDNAs have been recently characterized.

These cDNAs have been combined either with a constitutively expressed CaMV35S promoter, or with the endosperm-specific rice Gt1 glutelin promoter (Okita et al 1989). As a selectable marker for the identification of transgenic plants, a hygromycin phosphotransferase (*hpt*) gene under the control of a CaMV35S promoter has been linked to these constructs.

Subsequently, precultured immature embryos of japonica rice variety Taipei 309 were bombarded with a homemade particle inflow device (Finer et al 1992). A total of 203 hygromycin-resistant plants transformed with cDNAs for either phytoene synthase, phytoene desaturase, or cotransformed with both has been recovered from these experiments. Fifty-nine of these plants contained a DNA fragment integrated into the genome of the correct size to represent the expected cDNAs.

Among these transgenic plants, several lines have been identified that accumulated high levels of phytoene in the endosperm of the mature seed. Reverse phase high performance liquid chromatography revealed phytoene levels up to 0.74 µg phytoene per gram dry weight. Accumulation of ζ-carotene, the product of phytoene desaturase, has not yet been demonstrated. Correct integration and expression of the phytoene synthase transgene was further confirmed by Western blot analysis and RT-PCR. Southern blot analysis of R_1 plants growing in the greenhouse showed stable inheritance of the transgenes from the R_0 to the R_1 generation. Mendelian segregation patterns furthermore suggest a simple integration pattern in a single locus on one chromosome. Biochemical analysis of the R_1 plants will be performed as soon as seeds are available.

Cited references

Armstrong GA, Alberti M, Leach F, Hearst JE. 1989. Nucleotide sequence, organization, and nature of the protein products of the carotenoid gene cluster of *Rhodobacter capsulatus*. Mol. Gen. Genet. 216:254-268.

Bartley GE, Vitanen PV, Pecker I, Chamovitz D, Hirschberg J, Scolnik PA. 1991. Molecular cloning and expression in photosynthetic bacteria of soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. Proc. Natl. Acad. Sci. USA 88:6532-6536.

Buckner B. 1993. Molecular characterization of the *y1* genes of maize and rice. In: Research report, Rockefeller Foundation Grant RF 90031, #123.

Finer JJ, Vein P, Jones MW, McMullen MD. 1992. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep. 11:323-328.

Fray RG, Grierson D. 1993. Identification and genetic analysis of normal and mutant phytoene synthase of tomato by sequencing, complementation, and cosuppression. Plant Mol. Biol. 22:589-602.

Humphrey JH, West Jr KP, Sommer A. 1992. Vitamin A deficiency and attributable mortality among under 5 year olds. Bull. WHO 70(2):225-232.

Linden H, Misawa N, Saito T, Sandmann G. 1994. A novel carotenoid biosynthesis gene coding for z-carotene desaturase: functional expression, sequence, and phylogenetic origin. Plant Mol. Biol. 24:369-379.

Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izawa Y, Nakamura K, Harashima K. 1990. Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. J. Bacteriol. 172:6704-6712.

Okita TW, Hwang YS, Hnilo J, Kim WT, Aryan AP, Larson R, Krishnan HB. 1989. Structure and expression of the rice glutelin multigene family. J. Biol. Chem. 264:12573-12581.

Penteado MVC, Almeida LB. 1988. Occurrence of carotenoids in roots of five cultivars of cassava (*Manihot esculenta* Crantz) from Sao Paulo. Rev. Farm. Bioquim. Univ. S. Paulo 24:39-49.

Ray J, Bird CR, Maunders MJ, Grierson D, Schuch W. 1987. Sequence of pTOM5, a ripening related to cDNA from tomato. Nucleic Acids Res. 15:1057.

The Rockefeller Foundation. 1993. Potential for carotenoid biosynthesis in rice endosperm. Workshop report.

West Jr KP, Howard GR, Sommer A. 1989. Vitamin A and infection: public health implications. Annu. Rev. Nutr. 9:63-86.

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Citation: [IRRI] International Rice Research Institute. 1996. Rice genetics III. Proceedings of the Third International Rice Genetics Symposium, 16-20 Oct 1995. Manila (Philippines): IRRI.

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13. The AAPK coding sequence was amplified by PCR from the AAPK cDNA clone with the primers 5′-GAATCTCCACTACGACGCCGTTTACTTCCG-3′ and 5′-CCGTGCAACCATGGATATGGCATATACAAT-3′. The pAAPK-GFP construct was created by inserting (via Nco I digestion) the amplified AAPK coding sequence downstream of the 35S promoter and upstream of, and in frame with, the GFP coding sequence in the GFP expression vector (pGFP) described in (*17*). To create pAAPK(K43A)-GFP, the lysine at position 43 in the AAPK coding sequence was substituted with an alanine by site-directed mutagenesis [overlapping PCR method; [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989)]]. Constructs were sequenced to confirm correct junction, orientation, and/or site mutation.
14. Fifteen million *V. faba* guard cell protoplasts were transfected with pGFP, pAAPK-GFP, or pAAPK (K43A)-GFP by polyethylene glycol–mediated DNA transfer (*17*). Protoplasts were lysed and recombinant protein was immunoprecipitated with GFP peptide antibodies (Clontech, Palo Alto, CA) and protein A–Sepharose CL-4B (Amersham Pharmacia Biotech, Picataway, NJ). Immunoprecipitated proteins were assayed for kinase activity using histone III-S (Sigma) as substrate (*4*). No histone phosphorylation by the pGFP control immunoprecipitate was observed. Analogous to native AAPK (*4*), histone phosphorylation by the pAAPK-GFP immunoprecipitate was enhanced when the immunoprecipitate was isolated from ABA-treated guard cells. When the same experiment was performed with pAAPK(K43A)-GFP, histone phosphorylation was reduced.
15. S. K. Hanks and T. Hunter, *FASEB J.* **9**, 576 (1995); O. Cohen, E. Feinstein, A. Kimchi, *EMBO J.* **16**, 998 (1997); K. Diener *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9687 (1997).
16. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
17. W. Chiu *et al.*, *Curr. Biol.* **6**, 325 (1996).
18. J. Sheen, *Science* **274**, 1900 (1996).
19. *V. faba* leaves were bombarded with DNA-coated [pGFP, pAAPK-GFP, or pAAPK(K43A)-GFP] gold particles (BioRad, Hercules, CA) with the use of a particle delivery system 1000/He (BioRad, Hercules, CA) as described [J. Marc *et al.*, *Plant Cell* **10**, 1927 (1998)]. Stomatal complexes were then assayed for ABA-prevented opening or closure stimulated by ABA, CO_2, or darkness. Conditions for opening experiments were as described [S. M. Assmann and T. I. Baskin, *J. Exp. Bot.* **49**, 163 (1998)] except that the incubation solution was 10 mM MES, 30 mM KCl, pH 6.1 with or without 50 μM [±]-*cis,trans*-ABA. For closure experiments, transformed leaves were illuminated with 0.20 mmol m^{-2} s^{-1} white light for 2.5 hours to open stomata. Epidermal peels were then taken and placed in incubation solution under darkness or treated with 25 μM [±]-*cis,trans*-ABA, or with 700 parts per million (ppm) CO_2 for 1 hour.
20. Abaxial guard cell protoplasts were isolated as described [X.-Q. Wang, W.-H. Wu, S. M. Assmann, *Plant Physiol.* **118**, 1421 (1998)]. Whole-cell patch-clamp experiments were performed as previously established (*22*). Pipette solution contained 100 mM KCl, 50 mM tetraethylammonium, 2 mM $MgCl_2$, 6.7 mM EGTA-$(Tris)_2$, 3.35 mM $CaCl_2$, 10 mM Hepes, pH 7.1 (Tris), and 5 mM Mg-ATP. Bath solution contained 40 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM MES-Tris pH 5.6. Osmolalities were adjusted with sorbitol to 500 mosmol/kg (in the pipette) or 470 mosmol/kg (in the bath). Protein kinase inhibitor K252a (Calbiochem, La Jolla, CA) was prepared as a 2 mM stock in dimethyl sulfoxide (DMSO); DMSO controls showed no effect.
21. J. I. Schroeder, C. Schmidt, J. Sheaffer, *Plant Cell* **5**, 1831 (1993); A. Schwartz *et al.*, *Plant Physiol.* **109**, 651 (1995).
22. C. Schmidt, I. Schelle, Y.-J. Liao, J. I. Schroeder, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9535 (1995).
23. J. I. Schroeder and S. Hagiwara, *Nature* **338**, 427 (1989).
24. Supplemental data are available at *Science* Online at www.sciencemag.org/feature/data/1045645.shl.
25. M. Schwarz and J. I. Schroeder, *FEBS Lett.* **428**, 177 (1998).
26. F. Gosti *et al.*, *Plant Cell* **11**, 1897 (1999).
27. B. R. Loveys, *New Phytol.* **98**, 563 (1984).
28. J. Li, Y-R. Lee, S. M. Assmann, *Plant Physiol.* **116**, 785 (1998).
29. X.-Q. Wang and S. M. Assmann, data not shown.
30. We thank the following for their advice: R. Cyr (provision of GFP expression vector), C. Granger (bombardment experiments), S. Gilroy and S. Ritchie (GFP photography), L. Haubrick (Northern blotting), and J. A. Snyder (CO_2 experiments). We also thank W. Lane and the Harvard Microchemistry Facility for mass spectrometric sequencing. Supported by NSF grant MCB-9874438.

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Engineering the Provitamin A (β-Carotene) Biosynthetic Pathway into (Carotenoid-Free) Rice Endosperm

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Rice (*Oryza sativa*), a major staple food, is usually milled to remove the oil-rich aleurone layer that turns rancid upon storage, especially in tropical areas. The remaining edible part of rice grains, the endosperm, lacks several essential nutrients, such as provitamin A. Thus, predominant rice consumption promotes vitamin A deficiency, a serious public health problem in at least 26 countries, including highly populated areas of Asia, Africa, and Latin America. Recombinant DNA technology was used to improve its nutritional value in this respect. A combination of transgenes enabled biosynthesis of provitamin A in the endosperm.

Vitamin A deficiency causes symptoms ranging from night blindness to those of xerophthalmia and keratomalacia, leading to total blindness. In Southeast Asia, it is estimated that a quarter of a million children go blind each year because of this nutritional deficiency (*1*). Furthermore, vitamin A deficiency exacerbates afflictions such as diarrhea, respiratory diseases, and childhood diseases such as measles (*2, 3*). It is estimated that 124 million children worldwide are deficient in vitamin A (*4*) and that improved nutrition could prevent 1 million to 2 million deaths annually among children (*3*). Oral delivery of vitamin A is problematic (*5, 6*), mainly due to the lack of infrastructure, so alternatives are urgently required. Success might be found in supplementation of a major staple food, rice, with provitamin A. Because no rice cultivars produce this provitamin in the endosperm, recombinant technologies rather than conventional breeding are required.

Immature rice endosperm is capable of synthesizing the early intermediate geranylgeranyl diphosphate, which can be used to produce the uncolored carotene phytoene by expressing the enzyme phytoene synthase in rice endosperm (*7*). The synthesis of β-carotene requires the complementation with three additional plant enzymes: phytoene desaturase and ζ-carotene desaturase, each catalyzing the introduction of two double bonds, and lycopene β-cyclase, encoded by the *lcy* gene. To reduce the transformation effort, a bacterial carotene desaturase, capable of introducing all four double bonds required, can be used.

We used *Agrobacterium*-mediated transformation to introduce the entire β-carotene biosynthetic pathway into rice endosperm in a single transformation effort with three vectors (Fig. 1) (*8*). The vector pB19hpc combines the sequences for a plant phytoene synthase (*psy*) originating from daffodil (*9*) (*Narcissus pseudonarcissus*; GenBank accession number X78814) with the sequence coding for a bacterial phytoene desaturase (*crtI*) originating from *Erwinia uredovora* (GenBank accession number D90087) placed under control of the endosperm-specific glutelin (Gt1) and the constitutive CaMV (cauliflower mosaic virus) 35*S* promoter, respectively. The phytoene synthase cDNA contained a 5′-sequence coding for a functional transit peptide (*10*), and the *crtI* gene contained the transit peptide (*tp*) sequence of

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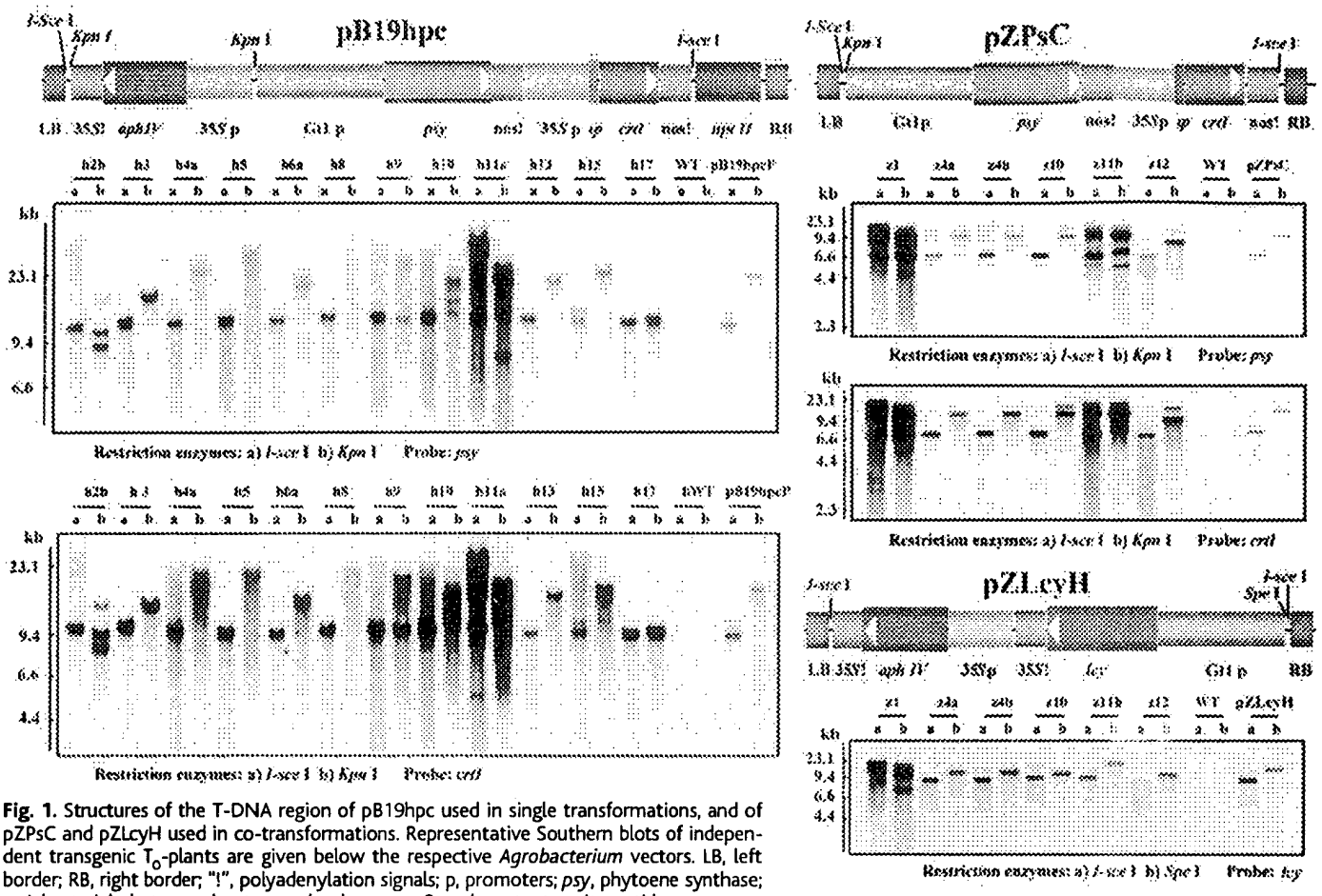

Fig. 1. Structures of the T-DNA region of pB19hpc used in single transformations, and of pZPsC and pZLcyH used in co-transformations. Representative Southern blots of independent transgenic T_0-plants are given below the respective *Agrobacterium* vectors. LB, left border; RB, right border; "!", polyadenylation signals; p, promoters; *psy*, phytoene synthase; *crtI*, bacterial phytoene desaturase; *lcy*, lycopene β-cyclase; *tp*, transit peptide.

the pea Rubisco small subunit (*11*). This plasmid should direct the formation of lycopene in the endosperm plastids, the site of geranylgeranyl-diphosphate formation.

To complete the β-carotene biosynthetic pathway, we co-transformed with vectors pZPsC and pZLcyH. Vector pZPsC carries *psy* and *crtI*, as in plasmid pB19hpc, but lacks the selectable marker *aphIV* expression cassette. Vector pZLcyH provides lycopene β-cyclase from *Narcissus pseudonarcissus* (*12*) (GenBank accession number X98796) controlled by rice glutelin promoter and the *aphIV* gene controlled by the CaMV 35*S* promoter as a selectable marker. Lycopene β-cyclase carried a functional transit peptide allowing plastid import (*10*).

Precultured immature rice embryos (n = 800) were inoculated with *Agrobacterium* LBA4404/pB19hpc. Hygromycin-resistant plants (n = 50) were analyzed for the presence of *psy* and *crtI* genes (Fig. 2). Meganuclease I–Sce I digestion released the ~10-kb insertion containing the *aphIV*, *psy*, and *crtI* expression cassettes. Kpn I was used to estimate the insertion copy number. All samples analyzed carried the transgenes and revealed mostly single insertions.

Immature rice embryos (n = 500) were inoculated with a mixture of *Agrobacterium* LBA4404/pZPsC and LBA4404/pZLcyH. Co-transformed plants were identified by Southern hybridization, and the presence of pZPsC was analyzed by restriction digestion. Presence of the pZLcyH expression cassettes was determined by probing I-Sce I–and Spe I–digested genomic DNA with internal *lcy* fragments. Of 60 randomly selected regenerated lines, all were positive for *lcy* and 12 contained pZPsC as shown by the presence of the expected fragments: 6.6 kb for the I-Sce I–excised *psy* and *crtI* expression cassettes from pZPsC and 9.5 kb for the *lcy* and *aphIV* genes from pZCycH (Fig. 1). One to three transgene copies were found in co-transformed plants. Ten plants harboring all four introduced genes were transferred into the greenhouse for setting seeds. All transformed plants described here showed a normal vegetative phenotype and were fertile.

Mature seeds from T_0 transgenic lines and from control plants were air dried, dehusked, and, in order to isolate the endosperm, polished with emery paper. In most cases, the transformed endosperms were yellow, indicating carotenoid formation. The pB19hpc single transformants (Fig. 2A) showed a 3:1 (colored/noncolored) segregation pattern, whereas the pZPsC/pZLcyH co-transformants (Fig. 2B) showed variable segregation. The pB19hpc single transformants, engineered to synthesize only lycopene (red), were similar in color to the pZPsC/pZLcyH co-transformants engineered for β-carotene (yellow) synthesis.

Seeds from individual lines (1 g for each line) were analyzed for carotenoids by photometric and by high-performance liquid chromatography (HPLC) analyses (*13*). The carotenoids found in the pB19hpc single transformants accounted for the color; none of these lines accumulated detectable amounts of lycopene. Instead, β-carotene, and to some extent lutein and zeaxanthin, were formed (Fig. 3). Thus, the lycopene α(f)- and β-cyclases and the hydroxylase are either constitutively expressed in normal rice endosperm or induced upon lycopene formation.

The pZPsC/pZLcyH co-transformants had a more variable carotenoid pattern ranging from phenotypes similar to those from single transformations to others that contain β-carotene as almost the only carotenoid. Line z11b is such an example (Fig. 3C and Fig. 2B, panel 2) with 1.6 μg/g carotenoid in the endosperm. However, reliable quantitations must await homozygous lines with uniformly colored grains. Considering that extracts from the sum of (colored/

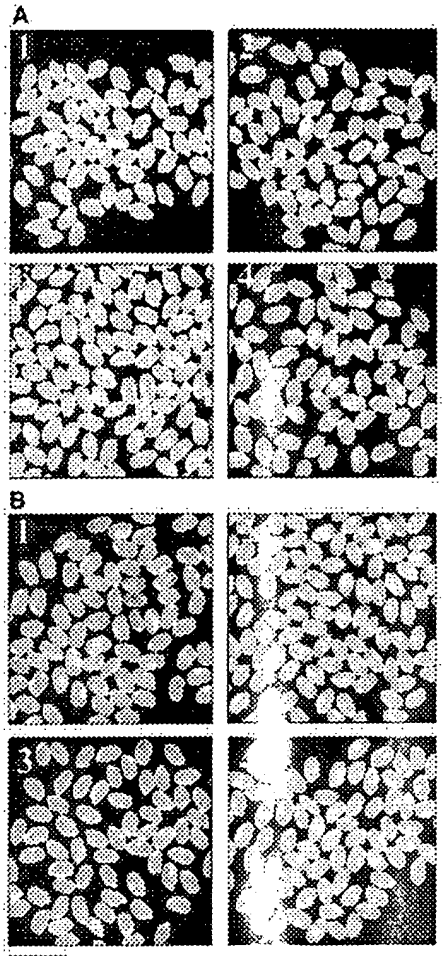

Fig. 2. Phenotypes of transgenic rice seeds. Bar, 1 cm. (**A**) Panel 1, untransformed control; panels 2 through 4, pB19hpc single transformants lines h11a (panel 2), h15b (panel 3), h6 (panel 4). (**B**) pZPsC/pZLcyH co-transformants lines z5 (panel 1), z11b (panel 2), z4a (panel 3), z18 (panel 4).

noncolored) segregating grains were analyzed, the goal of providing at least 2 μg/g provitamin A in homozygous lines (corresponding to 100 μg retinol equivalents at a daily intake of 300 g of rice per day), seems to be realistic (*7*). It is not yet clear whether lines producing provitamin A (β-carotene) or lines possessing additionally zeaxanthin and lutein would be more nutritious, because the latter have been implicated in the maintenance of a healthy macula within the retina (*14*).

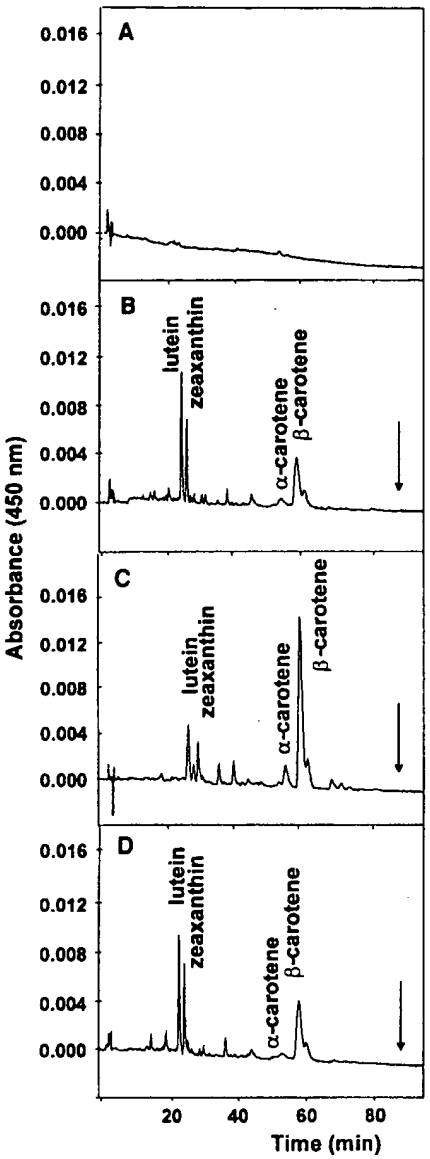

Fig. 3. The carotenoid extracts from seeds (1 g for each line) were subjected quantitatively to HPLC analysis. (**A**) Control seeds, (**B**) line h2b (single transformant), (**C**) line z11b (co-transformant), and (**D**) z4b (co-transformant). The site of lycopene elution in the chromatogram is indicated by an arrow.

References and Notes

1. A. Sommer, *J. Nutr.* **119**, 96 (1988).
2. J. P. Grant, *The State of the World's Children* (Oxford Univ. Press, Oxford, 1991).
3. K. P. West Jr., G. R. Howard, A. Sommer, *Annu. Rev. Nutr.* **9**, 63 (1989).
4. J. H. Humphrey, K. P. West Jr., A. Sommer, *WHO Bull.* **70**, 225 (1992).
5. A. Pirie, *Proc. Nutr. Soc.* **42**, 53 (1983).
6. A. Sommer, in *Elevated Dosages of Vitamins: Benefits and Hazards*, P. Walter, G. Erubacher, H. Stähelin, Eds. (Hans Huber, Toronto, Canada, 1989), pp. 37–41.
7. P. Burkhardt *et al.*, *Plant J.* **11**, 1071 (1997).
8. Three vectors—pUC18, pPZP100, and pBin19 (*15–17*)—were digested with Eco RI and Hind III and a synthetic linker flanking by meganuclease I-Sce I including Kpn I, Not I, and Sma I (5′-AATTCATTACCCTGTTATCCCTACCCGGGCGGCCGCGGTACCATTACCCTGTTATCCCTAA-3′) and (5′-AGCTTTAGGGATAACAGGGTAATGGTACCGCGGCCGCCCGGGTAGGGATAACGGGTAATG-3′) were introduced, forming pUC18M, pPZP100M, and pBin19M, respectively. An intermediate vector was made by insertion of the *crtI* expression cassette excised from Hind III/Eco RI–digested pUCET4, originally derived from pYPIET4 (*11*), into pBluescriptKS with Hind III/Eco RI digestion, followed by insertion of *psy* expression cassette from Sac II–blunted/Kpn I–digested pGt1psyH (*7*) into the Kpn I/Xho I–blunted previous vector. Finally, *crtI* and *psy* expression cassettes were isolated with Kpn I/Not I digestion and inserted into Kpn I/Not I–digested pUC18M and designated as pBaal3. pBin19hpc was made by insertion of a Kpn I fragment originally from pCIB900 (*18*) containing *aphIV* selectable marker gene into pBaal3, followed by digestion of the I-Sce I fragment of the resulting plasmid and insertion into I-Sce I–digested pBin19M. pZPsC was obtained by insertion of the I-SceI fragment of pBaal3 bearing the *psy* and *crtI* genes into I-Sce I–digested pPZP100M. pZLcyH was constructed by digestion of pGt1LcyH with I-Sce I and insertion of the resulting fragment, carrying *lcy* and *aphIV*, into I-Sce I–restricted pPZP100M. The three vectors were separately electroporated into *Agrobacterium tumefaciens* LBA4404 (*19*) with corresponding antibiotic selection. Callus induction: Immature seeds of japonica rice cultivar TP 309 at milk stage were collected from greenhouse-grown plants, surface-sterilized in 70% ethanol (v/v) for 1 min, incubated in 6% calcium hypochloride for 1 hour on a shaker, and rinsed three to five times with sterile distilled water. Immature embryos were then isolated from the sterilized seeds and cultured onto NB medium [N6 salts and B5 vitamins, supplemented with 30 g/l maltose, 500 mg/l proline, 300 mg/l casein hydrolate, 500 mg/l glutamine, and 2 mg/l 2,4-D (pH 5.8)]. After 4 to 5 days, the coleoptiles were removed, and the swelled scutella were subcultured onto fresh NB medium for 3 to 5 days until inoculation of *Agrobacterium*. Transformation: 1-week-old precultured immature embryos were immersed in *Agrobacterium tumefaciens* LBA 4404 cell suspension as described (*20*). For co-transformation, LBA4404/pZPsC [optical density at 600 nm (OD_{600}) = 2.0] mixed with an equal volume of LBA4404/pZLcyH (OD_{600} = 1.0) was used for inoculation after acetonsyringone induction. The inoculated precultured embryos were co-cultivated onto NB medium supplemented with 200 mM acetonsyringone for 3 days, subcultured on recovery medium (NB with 250 mg/l cefotaxime) for 1 week and then transferred onto NB selection medium in the presence of 30 mg/l hygromycin and 250 mg/l cefotaxime for 4 to 6 weeks. Transgenic plants were regenerated from recovered resistant calli on NB medium supplemented with 0.5 mg/l NAA and 3 mg/l BAP in 4 weeks, rooted and transferred into the greenhouse.
9. M. Schledz *et al.*, *Plant J.* **10**, 781 (1996).
10. M. Bonk *et al.*, *Eur. J. Biochem.* **247**, 942 (1997).
11. N. Misawa *et al.*, *Plant J.* **4**, 833 (1993).
12. S. Al-Babili, E. Hobeika, P. Beyer, *Plant Physiol.* **112**, 1398 (1996).
13. Dehusked seeds were polished for 6 hours with emery paper on a shaker. The endosperm obtained was ground to a fine powder and 1 g was extracted repeatedly with acetone. Combined extracts were used to record the ultraviolet-visible spectrum, allowing quantification using ε_{450nm} 134,000 l^{-1} mol^{-1} cm^{-1} for β-carotene. The samples were dried and the residue quantitatively applied in 30 μl chloroform to HPLC for analysis using a photodiode array detector (Waters) and a C_{30} reversed-phase column (YMC Europe GmbH) with the solvent system A [methanol: *tert*-butylmethyl ether (9:1, v/v)] and system B [methanol:*tert*-butylmethyl ether:H_2O (6:1.2:1.2, v/v/v)], using a gradient of 100% B to 43% B within 25 min, then to 0% B within a further 75 min. Final conditions were maintained for an additional 10 min. Photometric quantifications were re-examined by HPLC using synthetic all-*trans* lycopene as an external standard.
14. J. T. Landrum *et al.*, *Exp. Eye Res.* **65**, 57 (1997).
15. C. Yanish-Perron, J. Vieira, J. C. Messing, *Gene* **33**, 103 (1985).
16. P. Hajdukiewicz *et al.*, *Plant Mol. Biol.* **25**, 989 (1994).
17. M. Bevan, *Nucleic Acids Res.* **12**, 8711 (1984).
18. J. Wuenn *et al.*, *Bio/Technology* **14**, 171 (1996).
19. A. Hoekema, P. R. Hirsch, P. J. J. Hooykaas, R. A. Schilperoort, *Nature* **303**, 179 (1983).
20. M. Uze *et al.*, *Plant Sci.* **138**, 87 (1997).

21. The *crtI* gene fused to the transit peptide sequence was kindly provided by N. Misawa (Kirin Co., Ltd., Japan). We thank V. Ducrey and P. Burkhardt for their valuable contributions, S. Schauer, K. Konja, and U. Schneider-Ziebert for skillful technical assistance, and R. Cassada for correcting the English version of the manuscript. Supported by the Rockefeller Foundation (1993–1996), the European Community Biotech Program (FAIR CT96, 1996–1998) (P.B.), the Swiss Federal Office for Education and Science (I.P.) and by the Swiss Federal Institute of Technology (1993–1996).

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The regulation and genetic manipulation of carotenoid biosynthesis in tomato fruit

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Abstract: The Ailsa Craig variety of tomato (*Lycopersicon esculentum*) has been transformed with carotenoid genes from higher plants and bacteria. Progeny have been analysed for their carotenoid levels, carotenogenic enzyme activities and levels of gene expression. Ultrastructural studies have revealed changes in plastid structure. A similar approach has also been adopted with the high pigment (*hp*) mutant variety, which has elevated levels of carotenoids compared with the parental cultivar.

INTRODUCTION

The ripening of tomato (*Lycopersicon esculentum*) fruit is a highly regulated process during which the colour, flavour, aroma and texture change in a coordinated manner. One of the most noticeable characteristics of ripening is the dramatic increase in the carotenoid content of the fruit (ref. 1). The change in pigmentation is due to a massive accumulation of lycopene within the plastids and the disappearance of chlorophyll. The chloroplasts of mature green fruit change into chromoplasts, which contain lycopene in membrane-bound crystals (ref. 2). Early biochemical studies on tomato varieties were the basis on which the desaturase pathway from phytoene to lycopene was established (ref. 3). The carotenogenic enzymes of higher plants are located within the plastid (ref. 4), but their encoding genes are nuclear (ref. 5).

Analysis of tomato fruit, at 5 stages of development and ripening, has shown that the greatest carotenogenic activities are in green fruit, with phytoene synthase being located in the plastid stroma, whereas phytoene metabolism is associated with the plastid membranes (ref. 6).

In order to modify carotenoid levels in the tomato and to understand the regulatory mechanisms that control the formation and deposition of carotenoids in tomato fruit, we have used two approaches. Firstly, a study of the high pigment (*hp*) mutant of tomato which has elevated levels of carotenoids (ref. 7) and, secondly, production of transgenic varieties which overexpress either the tomato phytoene synthase (*Psy*) cDNA (ref. 8), or the *crtI* (phytoene desaturase) gene from *Erwinia uredovora* (ref. 9).

Tomato has been chosen for several reasons. Firstly, the several-hundred-fold increase in carotenoids during ripening and the rapid transition from mature green to red ripe fruit facilitates measurements of pigment levels and gene expression. Secondly, tomato is easily modified by genetic techniques. Thirdly, it is a very important food crop with major use in the processing and the fresh market sectors.

CHARACTERISATION OF THE HIGH PIGMENT MUTANT

Analysis of the pigments in *hp*, in comparison to the Ailsa Craig parental strain, showed that the pericarp of both green and ripe fruit of the former contained increased levels of carotenoids (Table 1), showing that the mutation affects both chloroplast-containing and chromoplast-containing fruit.

TABLE 1. Carotenoids levels in pericarp of Ailsa Craig and *hp* mutant varieties

Variety	Carotenoid content (μg/g fresh weight)	
	Green	Ripe
Ailsa Craig	18.2	173
hp	29.1	275

TABLE 2. Major carotenoids of ripe fruit pericarp of Ailsa Craig and *hp* varieties

Variety	Carotenoid (% total)					
	Xanth	Lyc	β-C	δ-/γ-C	PF	P
Ailsa Craig	5.0	60.1	20.2	2.9	1.7	8.0
hp	5.2	59.8	20.2	2.8	1.1	8.1

δ-/γ-C, δ- and γ-carotenes; β-C, β-carotene; PF phytofluene; P, phytoene; Lyc, lycopene; Xanth, xanthophylls

Further analysis of the pericarp carotenoids of ripe fruit by HPLC revealed a very similar pattern of individual carotenoids in each cultivar (Table 2), indicating that the mutation is within a regulatory gene.

Measurement of phytoene synthase and desaturase enzyme activities *in vitro*, using standard protocols (ref.6) revealed little difference, on a specific activity basis, between green and ripe fruit. Therefore the increased levels of carotenoids (Table 1) cannot be accounted for by higher levels of carotenogenic enzymes.

Light microscopy of the ripe fruit pericarp, however, revealed a key difference between the two varieties. Although the size of chromoplasts was the same in each variety, the number of plastids per cell was some two-fold more in the *hp* mutant (1140 in *hp*, 540 in Ailsa Craig).

In conclusion, therefore, the mutation in *hp* that causes an increase in carotenoid levels in ripe fruit has affected the number of plastids per cell, thus increasing the amount of carotenoid that can accumulate in the tissues.

MODIFICATION OF TOMATO CAROTENOID LEVELS BY TRANSFORMATION

Choice of genes vector, construction and transformation

Phytoene synthase (Psy)

Earlier studies had shown that the phytoene synthase gene is strongly expressed during fruit ripening, with an increase in enzyme activity at the breaker stage (ref. 6). Therefore, we ligated the *Psy* cDNA (ref. 8) into a pUC18-derived vector p JR1 to produce p JRex5 under the control of the CaMV 358 constitutive promoter. The vector was transferred to *Agrobacterium tumefaciens* LBA 4404 by triparental mating, tomato stem segments were transformed and transgenic lines selected on the basis of kanamycin resistance.

Phytoene desaturase (crt I)

The *crt I* gene of *Erwinia uredovora*, linked to the pea Rubisco small subunit transit sequence and under the control of the CaMV promoter had been used previously to transform tobacco, resulting in elevated carotenoid levels in leaves (ref. 10). We have used the same construct to transform tomato stem explants and have selected transformants, as described above.

Analysis of transgenic cultivars

Phytoene synthase transformants

The primary transformants produced a range of phenotypes. Some 40% produced yellow fruit, containing very low levels of carotenoids comparable to those in *Psy* antisense fruit (ref. 11).

This effect is probably due to co-suppression (gene silencing). Other transformants exhibited unscheduled carotenoid deposition in whole plant tissue as well as immature fruit (ref. 11). The consequence of this was the accumulation of lycopene in mature green fruit (Table 3). However, the final levels of lycopene in the red ripe fruit were lower than in control tissue, suggesting that premature deposition of lycopene limits its accumulation during ripening. Plastid number had not changed in the transformants, but in co-suppressed fruit the plastids were clustered around the nucleus. The reason for this is currently unknown.

TABLE 3. Overexpression of phytoene synthase: effect on carotenoids in green and ripe fruit

	Total	Lycopene
	μg/g fresh weight	
Control fruit		
Mature green	6.3	0
Ripe (14dpb)	126	65
Overexpressing line		
Mature green	14	1.6
Ripe (14dpb)	60	34

dpb, days post breaker

Phytoene desaturase (crt I) transformants

Primary transformants exhibited three different colour phenotypes: red, orange-red and orange. No unscheduled colouration was observed, nor any co-suppression effects. Analysis of total carotenoid levels and individual pigments in ripe fruit revealed that there was a modest change in total carotenoids compared to the untransformed control (372 and 315 μg/g fresh weight, respectively), but the profile had changed with an increase in β-carotene levels in transformants (Table 4).

The transgene was stably inherited and homozygous progeny maintained the same colour phenotype as primary transformants. The progeny were also resistant to the bleaching herbicide, norflurazon, as found for the transformants of tobacco (ref. 10).

In conclusion,constitutive expression of the *Erwinia crtI* gene caused an accumulation of β-carotene in ripe fruit, especially the columella. The reasons for this change are currently under investigation. Preliminary results with an antibody to the CrtI protein have shown that its level is higher in ripening fruit of the transformants than in the control fruit.

TABLE 4. Overexpression of *crt I*: effect on carotenoid levels in ripe fruit

	Total	Xanth	Lyc	β-C	γ-/δ-C	PF	P
	µg/g FW	(% total)					
Ailsa Craig							
Pericarp	183	1.5	76	10	tr	2.2	3.9
Columella	119	2.5	44	40	5.0	0.7	2.8
Jelly	13	8.9	17	68	0.3	tr	tr
Crt I transformant							
Pericarp	241	0.7	73	23	0.3	tr	tr
Columella	95	14	23	57	4.3	1.2	tr
Jelly	36	2.8	11	73	0.2	2.2	tr

See Table 2 for abbreviations

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REFERENCES

1. D. Laval-Martin, J. Quennemet and R. Monéger. *Phytochemistry* **14**, 2357-2362 (1975)
2. W.M. Harris and A.R. Spurr. *Am. J. Bot.* **56**, 369-379 (1969)
3. J.W. Porter and R.E. Lincoln. *Arch. Biochem. Biophys.* **27**, 390-403 (1950)
4. K. Kreuz, P. Beyer and H. Kleinig. *Planta* **154**, 66-69 (1982)
5. J.T.O. Kirk and R. Tilney-Bassett. *The Plastids*. Freeman, San Francisco (1978)
6. P.D. Fraser, M.R. Truesdale, C.R. Bird, W. Schuch and P.M. Bramley. *Plant Physiol.* **105**, 403-413 (1994)
7. C.D. Clayberg, L. Butler, C.M. Rick and P.A. Young. *J. Hered.* **51**, 167-174 (1960)
8. J. Ray, P. Moreau, C. Bird, A. Bird, D. Grierson, M. Maunders, M. Truesdale, P.M. Bramley and W. Schuch. *Plant Mol. Biol.* **119**, 401-404 (1992)
9. N. Misawa, M. Nakagawa, K. Kabayashi, S. Yamano, Y. Izawa, K. Nakamura and K. Harashima. *J. Bacteriol.* **172**, 6704-6712 (1990)
10. N. Misawa, S. Yamano, H. Linden, M.R. de Filipe, M. Lucas, H. Ikenaga and G. Sandmann. *Plant J.* **4**, 833-840 (1993)
11. M.R. Truesdale. *PhD Thesis*, University of London (1995)

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Golden Rice and Beyond

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EMOTIONS ARE THE PROBLEM, NOT RATIONAL DISCOURSE

The term "golden rice" was coined by a Thai businessman who is active in initiatives aimed at reducing the birth rate, a major cause of the food security problem. As it turned out, the term "golden rice" has proven to be enormously successful in piquing the interest of the public. (I gave up tallying its mention in the popular media after more than 30 television broadcasts and 300 newspaper articles, but I am still busy with requests for interviews every week.) It is difficult to estimate how much of its celebrity stems from its catchy moniker and how much is from the technological breakthrough it represents. Needless to say, we live in a society that is strongly influenced (not to say manipulated) by the media. As the popular media live by selling news, "catchy" names are especially useful in attracting the interest of media consumers. The "story," however, must also be accompanied by an important message, in this case, that the purely altruistic use of genetic engineering technology has potentially solved an urgent and previously intractable health problem for the poor of the developing world. And this is my first message and my response to Chris Somerville's (2000) contribution: I, too, believe in the power of education and rational discourse. However, after more then 10 years on the frontlines of the public debate concerning genetically modified organisms (GMOs), I have learned that even with the help of the media, rational arguments succeed in influencing only a small minority of the public-at-large. In short, rational arguments are poor ammunition against the emotional appeals of the opposition. The GMO opposition, especially in Europe, has been extraordinarily successful in channeling all negative emotions associated with the supposed dangers of all new technologies as well as economic "globalization" onto the alleged hazards presented by the release of GMOs into the food chain. This is one reason why the story of "golden rice" is so important: In the short history of GMO research, "golden rice" is unique in having been embraced by the public-at-large. The reason for this, I believe, lies in its emotional appeal: People are truly concerned about the fate of blind children, and they are willing to support a technology that offers the children at risk the opportunity to avoid blindness.

I fully agree with the opinion of Maarten Chrispeels (2000) that "food security" for developing countries is one of the major challenges for mankind. I believe that scientists, as a privileged group of citizens, have more than an academic responsibility to advance science: They must also accept a higher social responsibility and, wherever possible, use science to help solve the important problems not of industry, but of humanity. In this respect our scientific community is not in balance, and the public senses this intuitively. This, in turn, has made it easy for the GMO opposition to wage a war of propaganda against our work with arguments to the effect that we are only pretending to work for mankind, or are only satisfying our own egos, or are working merely for the profits of industry. For example, laypeople often ask if food security for developing countries is such a dire problem, and if scientists feel that GMO technology should be developed to contribute to a solution, then why are so many scientists working on Arabidopsis and so few on those plants that feed the poor? Of course, one can pontificate about the importance of basic research and how all the knowledge gained from Arabidopsis will ultimately expedite the improvement of major crops, but one realizes that the average citizen remains emotionally unswayed by such arguments. The public's skepticism is heightened by the fact that many scientists do have funds from industry and, therefore, have their sensiblities attuned to solutions of problems of interest to industry. Press releases from the agrobiotechnology industry relating to work on food security in developing countries are taken as disingenuous and serve only to foster ill will against the technology. So what can we do to improve the public sentiment about the technology? We need more examples of the "golden rice" type; namely, successful projects that were developed in public institutions using public funding that address an urgent need, are not solvable with traditional techniques, are being made available free of charge and limitations to the poor, and have no deleterious effects on the environment or human health.

GOLDEN RICE: THE SCIENTIFIC CHALLENGE

In the early 1990s, when we proposed to the Rockefeller Biotechnology Program (New York) to initiate a project to genetically engineer the provitamin-A pathway into the rice endosperm, we were fortunate that the Rockefeller Foundation had already had similar thoughts. The Foundation responded readily by

organizing a brainstorming session. The verdict of this initial session was that such a project had a low probability of success, but that it was worth trying because of its high potential benefit. That is how Peter Beyer (University of Freiburg, Germany) and I got together, and this collaboration turned out to represent an ideal combination of skills. Peter Beyer was studying the regulation of the terpenoid pathway in daffodil and was working on the isolation of those genes we would need to establish the pathway in rice endosperm, whereas I had the engineering technology and was naïve enough to believe that the project was feasible. Naïveté was an important component because all those with appropriate knowledge had cited numerous reasons for skepticism. Our research determined that the last precursor of the pathway in endosperm was geranlygeranyl-pyrrophosphate and, as a consequence, it theoretically should be possible to reach β-carotene via four enzymes: phytoene synthase, phytoene desaturase, ζ-carotene desaturase, and lycopene cyclase (Burkhardt et al., 1997). There were hundreds of scientific reasons why the introduction and coordinated function of these enzymes would not be expected to work. Those with the necessary scientific knowledge were right in not believing in the experiment. When we finally had "golden rice" I learned that even my partner, Peter Beyer, and the scientific advisory board of The Rockefeller Foundation, except for Ralph Quatrano, had not believed that it could work. This exemplifies the advantage of my ignorance and naïveté: With my simple engineering mind I was optimistic throughout and therefore carried the project through, even when Rockefeller stopped funding Peter Beyer's group. Altogether it took 8 years, but the first breakthrough came when Peter Burkhardt of my laboratory recovered phenotypically normal, fertile, phytoene synthase-transgenic rice plants, which produced good quantities of phytoene in their endosperm (Burkhardt et al., 1997). This demonstrated two important facts: It was possible to specifically divert the pathway toward β-carotene, and channeling a considerable amount of geranly geranyl-pyrrophosphate away from the other important pathways had no severe consequences on the physiology and development. Xudong Ye of my laboratory did the crucial experiment: cotransformation with two *Agrobacterium* strains containing all the necessary genes plus a selectable marker. The resulting yellow-colored endosperm contained provitamin A and other terpenoids of nutritional importance and to everybody's surprise demonstrated that it was possible to engineer the entire biochemical pathway (Ye et al., 2000). A further key figure in our research was Salim Al-Babili from Peter Beyer's group who supplied all the successful constructs. The highest provitamin-A-producing line contains enough provitamin A (1.6 $\mu g\ g^{-1}$ endosperm) to expect a positive effect in relieving vitamin-A deficiency, but of course this has to be tested with bioavailability and feeding studies. However, these cannot be performed with the few grams of rice we can produce in our containment greenhouse. This will require hundreds of kilograms, which can be produced only in the field, and field release is still a problem in Europe, as it is in developing countries. (We are faced with a strong political movement for a 10-year moratorium in Switzerland.)

GOLDEN RICE: THE CHALLENGE OF INTELLECTUAL PROPERTY RIGHTS (IPRs)

"Golden rice" was developed to prevent vitamin-A deficiency in the poor and disadvantaged of developing countries. To fulfill this goal it has to reach the subsistence farmers free of charge and restrictions. Peter Beyer had written up a patent application, and Peter and I were determined to make the technology freely available. Because only public funding was involved, this was not considered too difficult. The Rockefeller Foundation had the same concept and the Swiss Federal Institute of Technology (Zurich) supported it, but the European Commission had a clause in its financial support to Peter Beyer, stating that industrial partners of the "Carotene Plus" project, of which our rice project was a small part, would have rights to project results. (The framework [IV and V] of European Union [EU] funding forces public research into coalitions with industry and thus is responsible for two very questionable consequences: Public research is oriented toward problems of interest to industry, and public research is losing its independence.) We did not consider this to be too big a problem because the EU funding was only a small contribution at the end of the project, but we soon realized that the task of technology transfer to developing countries, the international patent application, and the numerous IPRs and technical property rigths (TPRs) we had used in our experiments were too much for two private persons to handle properly. We urgently needed a powerful partner (because of the deadline of the international patent application). In discussions with industry the definitions of "subsistence farmer" and "humanitarian use" were the most difficult problems to be solved. We wanted a definition as generous as possible, because we not only wanted the technology to be free for small-scale farmers, but we also wanted to contribute to poverty alleviation via local commercial development. It is very fortunate that the company that agreed to the most generous definition was also the company that had legal rights because of its involvement in the EU project. This facilitated the agreement, via a small licensing company (Greenovation, Freiburg, Germany), with Zeneca (Fernhurst, UK). Zeneca received an exclusive license for commercial use and in return supports the humanitarian use via the inventors for developing countries. The cutoff line between humanitarian and commercial use is $10,000 (income

from "golden rice"). This agreement also applies for all subsequent applications of this technology to other crop plants. It turned out that our agreement with Zeneca and the involvement of our partner in Zeneca, Adrian Dubock, were real assets in developing the humanitarian aspect of the project. Adrian was very helpful in reducing the frightening number of IPRs and TPRs. He also organized most of the free licenses for the relevant IPRs and TPRs such that we are now in the position of granting "freedom to operate" to those public research institutions in developing countries to proceed in introducing the trait into local varieties. Publicity sometimes can be helpful: Only a few days after the cover story about "golden rice" had appeared in *Time*, I had a phone call from Monsanto offering free licenses for the company's IPR involved.

MAKING BEST USE OF (NOT FIGHTING ABOUT) PATENTS HELPS THE POOR AND UNDERPRIVILEGED

At this point it is appropriate to add a more general comment on patents and the heavy opposition against patenting in life sciences. Because we did not know how many and which IPRs we had used in developing the "golden rice," and because further development for the humanitarian purpose required "freedom to operate" for the institutions involved, The Rockefeller Foundation commissioned an IPR audit through the International Service for the Acquisition of Agri-Biotech Applications. The outcome was shocking (International Service for the Acquisition of Agri-Biotech Applications brief nos. 20–2,000). There were 70 IPRs and TPRs belonging to 32 different companies and universities, which we had used in our experiments and for which we would need free licenses to be able to establish a "freedom to operate" situation for our partners, who were keen to begin further variety development. Because I was also blocked by an unfair use of a material transfer agreement, which had no causal relation to "golden rice" development, I was initially upset. It seemed to me unacceptable, even immoral, that an achievement based on research in a public institution and exclusively with public funding and designed for a humanitarian purpose was in the hands of those who had patented enabling technology earlier or who had sneaked in a material transfer agreement in the context of an earlier experiment. It turned out that whatever public research one was doing, it was all in the hands of industry (and some universities). At that time I was much tempted to join those who fight patenting. Upon further reflection, however, I realized that the development of "golden rice" was only possible because of the existence of patents. Much of the technology that I had been using was publicly available only because the inventors, by patenting, could protect their rights. Without patents, much of this technology would have remained secret. To take full advantage of available knowledge to benefit the poor, it does not make sense to fight against patenting. It makes far more sense to fight for a sensible use of IPRs. Thanks to public pressure there is much goodwill in the leading companies to come to an agreement on the use of IPR/TPR for humanitarian use that does not interfere with commercial interests of the companies. An interesting discussion of this issue was part of a recent satellite meeting associated with the World Food Prize Symposium 2000 in Des Moines, Iowa (for more information, contact C.S. Prakash, e-mail: prakash@acd.tusk.edu).

We are now in a situation in which we have verbal confirmation for free licenses for humanitarian use for all intellectual and technical property involved. To date, details cannot yet be disclosed because some IPR owners prefer anonymity. Thanks to the interest of the agbiotech companies to use "golden rice" for better acceptance of the GMO technology, and thanks to the pressure against GMOs built up by the opposition, the IPR situation was easier to solve than expected.

GOLDEN RICE: THE CHALLENGE OF TECHNOLOGY TRANSFER

Having overcome the scientific problems, and having achieved freedom to operate, leaves technology transfer as the next hurdle. This is a far bigger task that anyone having no personal experience should assume. "Golden rice" so far consists of a series of provitamin-A-producing laboratory lines (TP 309). The characters of these lines must be transferred to as many locally adapted varieties and ecotypes in as many rice-growing countries as quickly as possible, and this transfer has to be organized such that all rules and regulations concerning the handling and use of GMOs will be strictly followed. Although we have had requests from many institutions in many countries, we believed it would be unwise to start the technology transfer on too large a scale. To aid in this endeavor, we have established a "Golden Rice Humanitarian Board" to help make the right decisions and to provide secretarial support. Again, our decision to work with Zeneca was extremely helpful. Adrian Dubock was willing to care for the task of the secretary. We have additional invaluable help from Katharina Jenny from the Indo-Swiss Collaboration in Biotechnology (ETH Zurich), an institution jointly financed by the Indian Department of Biotechnology (DBT; New Delhi, India) and the Swiss Development Corporation (Bern, Switzerland). Golden rice will be introduced into India in the established organizational framework of the Indo-Swiss Collaboration in Biotechnology, which has 10 years of experience in technology transfer. Thanks to this situation and thanks to the strong commitment of the DBT and the Indian Council for Agricultural Research (New

Delhi, India), India will take a leading role and can serve as a model for other countries. The project will begin with a careful assessment of needs, an analysis and comparisons of the pros and cons of alternative measures, and setting a framework for the optimal and complementary use of "golden rice." Of course, there will be bioavailability, substantial equivalence, toxicology, and allergenicity assessments and we are grateful for offers from specialists to help. Careful socioeconomic and environmental impact studies will help to avoid any possible risk and make sure that the technology reaches the poor. Care will be taken that the material is given only to institutions that ensure proper handling according to rules and regulations. Traditional breeding will transfer the trait into locally best adapted lines, and again will make sure that varieties important to the poor will be used and not fashionable varieties for the urban middle class. There will be also direct de novo transformation into important varieties, and this will be done with Man selection (Lucca et al., 2000). It is fortunate that the World Bank, the Indian Council for Agricultural Research, and DBT will probably share the costs for this development in India. Agreements have been established with several institutions in Southeast Asia, China, Africa, and Latin America and as soon as the written confirmation of the "freedom to operate" is in the hands of the "Humanitarian Board," material will be transferred.

GOLDEN RICE: THE CHALLENGE OF THE GMO OPPOSITION

A scientific breakthrough promises to add an essential dietary component (provitamin A) to one of the major food staples of the poor and developing world. Against all expectations, "freedom to operate" for humanitarian use has been achieved, enabling us to provide this technology free of charge and limitations, via national and international public research institutions and local rice breeders to the subsistence farmers in developing countries. Numerous rice-growing countries have expressed great interest in embracing this novel opportunity to help reduce malnutrition, and there is the institutional organization and the technical expertise to further develop this technology within the rice-growing countries. Is there any problem left that could interfere with the exploitation of "golden rice" to the benefit of the poor and disadvantaged in developing countries? It is unfortunate that the answer is yes: Greenpeace (www.greenpeace.org) and associated GMO opponents regard "golden rice" as a "Trojan horse" that may open the route for other GMO applications. As a consequence, by their singular logic, the success of "golden rice" has to be prevented under all circumstances, irrespective of the damage to those for whose interest Greenpeace pretends to act. The strategy is simple and has proven effective in Europe: undermining the acceptance of the consumer.

"Golden rice" fulfills all the wishes the GMO opposition had earlier expressed in their criticism of the use of the technology, and it thus nullifies all the arguments against genetic engineering with plants in this specific example.

Golden rice has not been developed by or for industry.

It fulfills an urgent need by complementing traditional interventions.

It presents a sustainable, cost-free solution, not requiring other resources.

It avoids the unfortunate negative side effects of the Green Revolution.

Industry does not benefit from it.

Those who benefit are the poor and disadvantaged.

It is given free of charge and restrictions to subsistence farmers.

It does not create any new dependencies.

It will be grown without any additional inputs.

It does not create advantages for rich landowners.

It can be resown every year from the saved harvest.

It does not reduce agricultural biodiversity.

It does not affect natural biodiversity.

There is, so far, no conceptual negative effect on the environment.

There is, so far, no conceivable risk to consumer health.

It was not possible to develop the trait with traditional methods, etc.

Optimists might have expected, therefore, that the GMO opposition would have welcomed the advent of "golden rice." The GMO opposition, however, has been doing everything in its power to prevent "golden rice" from reaching subsistence farmers. This is because the GMO opposition has a hidden, political agenda. It is not so much the concern about the environment, or the health of the consumer, or the help for the poor and disadvantaged. It is a radical fight against a technology and for political success. This could be tolerated in rich countries where people have a luxurious life even without the new technology. However, it cannot be tolerated in poor countries, where the technology can make the difference between life and death or between health and severe illness.

However, because its acceptance has to be prevented under all circumstances, new arguments had to be invented. Thus, the oppposition has argued that there is no need for "golden rice" because distribution of synthetic vitamin A works perfectly, or that nobody wants it because it tastes awful, or that people who eat "golden rice" will lose their hair and sexual potential! If you are interested in further misinformation of this kind, please consult various anti-GMO Web sites on the Internet.

One is tempted to ignore these aspersions, but this would be the wrong strategy. I am afraid that Greenpeace's specious arguments against "golden rice" will lead to unwarranted opposition in some devel-

oping countries. The consequence will be millions of unnecessarily blind children and vitamin-A deficiency-related deaths. For these reasons, we have the moral obligation to enlighten the public concerning the dangerous and immoral game the GMO opposition is playing. Anti-GMO activists are using all their political power (and funds collected ostensibly to protect whales and baby seals) to prevent a humanitarian project aimed toward helping millions of people who are malnourished and in grave danger of going blind. The GMO opposition often demands that scientists be held responsible for their actions. At the same time, however, they sidestep responsibility for the harm they cause to the disadvantaged and poor with their creation of a most hostile atmosphere against GMOs in Europe and elsewhere. In my judgment, hindering a person's access to life- or sight-saving food is criminal. To do this to millions of children is so criminal that it should not be tolerated by any society. It is unfortunate that our society, especially in Europe, is unable to recognize the true face of an organization that is using the mask of a few idealists risking their lives to save a few whales. The extent to which Greenpeace can act outside the law with impunity, and how skewed the mind of a European judge can be, was recently demonstrated in a judicial court in Nottingham, UK. The vandalism by Greenpeace activists of a government-supported experimental plot examining the possible effects of transgenic maize on the environment was ruled justifiable because it had been done "in the higher interest of mankind." In my view, the Greenpeace management has but one real interest: to organize media-effective actions for fund raising. The "golden rice" case hopefully may help to unmask the true and shameful face of Greenpeace, but only if the media are willing to take them to task.

I share the optimism of Norman Borlaug (2000) concerning the potential that GMO technology has for improving the living conditions of the poor and underprivileged in developing countries. I admire his "standing up to the antiscience crowd." I wish that more internationally recognized personalities would demonstrate similar civil courage and that the scientific community (and the granting agencies) would find a bit more interest in contributing to solutions of the problems of food security. In the long run, our science has the best chance to survive if we win the support of the public. For this, it is no longer sufficient simply to do good science—we must also be activists for and popularizers of the new technology.

LITERATURE CITED

Borlaug NE (2000) Ending world hunger: the promise of biotechnology and the threat of antiscience zealotry. Plant Physiol **124:** 487–490

Burkhardt PK, Beyer P, Wünn J, Klöti A, Armstrong G, Schledz M, von Lintig J, Potrykus I (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. Plant J **11:** 1071–1078

Chrispeels MJ (2000) Biotechnology and the poor. Plant Physiol **124:** 3–6

Lucca P, Ye X, Potrykus I (2000) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. Mol Breed (in press)

Nash JM (2000) Grains of hope. Time, July 31, 2000, pp 38–46

Somerville C (2000) The genetically modified organism conflict. Plant Physiol **123:** 1201–1202

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science **287:** 303–305

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